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EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON MICROBIAL AMMONIA PRODUCTION IN BEDDING MATERIALS CONTAMINATED WITH LIVESTOCK URINE AND ON GROWTH INHIBITION OF UREOLYTC AND MASTITIS CAUSING BACTERIA

presented by

Amina R. Gaber

has been accepted towards fulfillment of the requirements for

Master degree in Animal Science

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Date April 28, 1997

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By

Amina R. Gaber

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Master in Science

Department of Animal Science

1997

ABSTRACT

EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON MICROBIAL AMMONIA PRODUCTION IN BEDDING MATERIALS CONTAMINATED WITH LIVESTOCK URINE AND ON GROWTH INHIBITION OF UREOLYTIC AND MASTITIS CAUSING BACTERIA

By

Amina R. Gaber

Simple technology is not available for quantitatively detecting and measuring ammonia production from livestock bedding. The effect of control (C), boric acid (B), pine oil (PO) and their combination (M) on ammonia production, growth of ureolytic and mastitis causing bacteria was studied in different bedding materials (sand, straw, corncobs, woodshaving, newspaper and sawdust) contaminated with livestock (cow, horse, pig) urine or chicken manure. Kitagawa tubes were proven to be suitable for semi-quantitative detection of ammonia. Urine and beddings are the main sources of ureolytic bacteria. Treatments showed different levels of inhibition depending on bedding used and urine source. In general (PO) did not affect ammonia production ($P > 0.5$), while (B) was effective in some cases ($P < 0.5$). The combination was the most effective ($P < 0.5$) treatment in reducing ammonia production, especially when used with sand. Urine characteristics affected ammonia production ($P < 0.001$). Since cow and horse urine contain high levels of urea they produced more ammonia than pig urine or chicken manure. Pine oil alone was not as effective as either boric acid or the combination in reducing growth of ureolytic or mastitis causing bacteria. In conclusion, the application of (M) to either sand or sawdust is efficient in reducing ammonia production.

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ACKNOWLEDGEMENT

It is with great pleasure and respect that I acknowledge the encouragement and support that I received from Dr. Melvin Yokoyama my major advisor, during the entire course of my study at Michigan State University. Dr. Mel always guided me and patiently read my numerous drafts and made excellent and valuable suggestions and remarks.

It is an honor to thank the members of my committee Drs. Robert Hausinger, Herb Bucholtz, Werner Bergen for their helpful suggestions, assistance and interest in the completion of this work.

I would like to thank Dr. Hogberg, the Chairman of the Animal Science Department for his support and assistance during my Masters degree. Also, my thanks is extended to Dr. Sue Hengemuehle, for being friendly and helpful at times of need. I would like to thank everyone in the department for giving me the opportunity to work in such a pleasant atmosphere.

My sincere thanks goes to Clorox company for its support, as well as, providing me with the necessary guidance that this work required.

To my parents, Mr. Ramzy Hassan Gaber and Mrs. Afaf Ibrahim I give them all my gratitude and appreciation for they taught me perservance and always placed a great value on the pursuit of education.

The demands of this work necessitated neglect of my duties towards my kids, Dina and Raafat, who always cheered me up and supported me; though sometimes they used to say " who told you to do this thing." Thanks guys for giving mommy the chance

to finish her thesis.

Last, but not least, I would like to thank my husband Dr. Ahmed Raafat though I do not have enough words to express my deepest and sincerest appreciation to him. This work would not have been possible without his tremendous and continuous support and motivation. Thanks Ahmed.

This is a major blemish in an otherwise worthwhile experience.

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LITERATURE REVIEW

In 1954, the first microbial urease was purified and characterized from the organism *Bacillus pasteurii* (Larson et al., 1954). Since then, numerous work have been done describing the purification of bacterial urease from different microbial species (Eng et al., 1986; Glemzha, 1986; Hausinger, 1986; Schneider et al., 1984 and Wong et al., 1974).

The most extensively studied urease is the jack bean plant enzyme, urease is also produced by over 200 species of bacteria (Baird et al., 1981; Kolmark, 1969; Mackay et al., 1982 and Sumner et al., 1947) as well as many eucaryotes. Urease from the jack bean plant *(Canavalia ensiformis*) has been well characterized at the biochemical level (Dixon et al., 1980a, 1980b, 1980c, Takashima et al., 1988). The molecular weight of the enzyme, first reported to be 480,000, has been shown to be 544,740 (Reithel et al., 1967). The enzyme is composed of six copies of a single subunit which has a molecular size of 90,790 daltons (Mamiya, 1985) and nickel as a cofactor (Dixon et al., 1975). Early work indicated that the enzyme was completely specific for the substrate urea (Sumner, 1951) and it has since been expanded to include hydroxyurea (Fishbein et al., 1965) dihydroxyurea (Fishbein, 1969), semicarbazide (Gazzola et al., 1973), formamide (Fishbein, 1977) and acetamide and N-methylurea (Dixon et al., 1980c).

There are number of reasons for the recent interest in bacterial ureases. First, in agriculture urease plays an important role in ammonia release from

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livestock buildings, which contributes to air pollution. Second, ureases are nickel metalloenzyme and they are studied as models for metallocenter biosynthesis (Hausinger, 1987, 1990). Third, urease genes are studied to gain a better understanding of their role in the utilization of nitrogen by bacteria (Collins et al., 1993; Friedrich and Magasanik, 1977 and Macaluso et al., 1990). Finally, interest in urease is due to the association of the enzyme with human disease.

AGRICULTURAL IMPORTANCE OF UREASE

Livestock odors are generally regarded as nuisance pollutants. As livestock production has moved to larger and more intense production units in recent years, odor complaints have increased from residents of surrounding communities. Complaints about livestock odors are a large proportion of the total number of complaints, since animal farms are located near residential districts. Hog and chicken operations are considered the main sources of odor pollution (Ritter, 1989). Microorganisms play an important role in ammonia production. Ammonia is the most highly reduced form of nitrogen occurring in nature and is produced by the anaerobic decomposition of proteins. Ammonia is a common odor around livestock buildings, feedlots and waste storage areas and its odor is familiar to most people. Its odor is commonly described as being sharp, pungent and somewhat irritating, whereas, amines have an odor similar to that of ammonia, but more severe and persistent (Miner et al., 1969).

Contaminated bedding is another major source of odor production. Many researchers have identified specific odor compounds of livestock waste odors. Barth et al., (1984) reported that more than 75 specific odorous compounds have been identified. These compounds are end products or intermediate products of biological reactions and include: volatile organic acids, alcohols, aldehydes, fixed gases, carbonyls, esters, amines, sulphides and nitrogen heterocyclics (Ritter, 1989). The management of waste is concerned mainly with how these odorous compounds are removed and stored and that is influenced by the quantity and nature of bedding material used.

ENVIRONMENTAL IMPORTANCE OF UREASE

Urease activity is widely found in soil and aquatic environments, where it plays an essential role in nitrogen metabolism (Bremner and Mulvaney, 1978). Agricultural production depends upon the fixed nitrogen which is available in the environment as macromolecules such as proteins and polynucleotides. In addition, effective urea fertilization requires controlled ureolysis to enhance the crop growth and minimize any damage. Proteins are hydrolyzed to amino acids and nucleotides by degradative enzymes. Further degradation of the amino acids especially arginine and the nucleotide monomers results in the production of urea, which is further broken down by urease to carbon dioxide and ammonia (Varner, 1959 and Vogels et al., 1976). Soil bacteria and plants utilize ammonia for anabolic nitrogen pathways, and as an energy source for nitrifying bacteria. The availability of endogenous fixed nitrogen sources in soil (e.g.

proteins and polynucleotides) is so low that fertilization is essential. Urea is widely used as a commercial fertilizer because it is low in cost, easy to handle, and has a high nitrogen content (Beaton, 1978). The United States produced 7.43 million tons of urea in 1987 (Reisch, 1988), which was used mainly in fertilizer products.

The use of urea as a fertilizer, however, does pose some agricultural problems. In some soils with a basic pH, up to 50% of the applied nitrogen is lost in the form of volatile ammonia (Mulvaney and Bremner, 1981 and Sahrawat, 1980). Additionally, uncontrolled urea hydrolysis results in elevated soil pH and ammonia toxicity for plants. In an effort to improve the use of applied urea, urease inhibitors are added to fertilizer urea. Preliminary results indicate that dihydric phenols and quinones effectively decrease urease activity in soil (Bremner and Douglas, 1971). Also, acetohydroxamic acid derivatives, which are specific urease inhibitors (Kobashi et al., 1971), will reduce the loss of nitrogen, as ammonia, from soil (Pugh and Waid, 1969) and therefore may serve as effective additives to fertilizers.

ODOR CONTROL

Considerable attention has been devoted to reducing the odor problem of livestock wastes. Complete elimination of odor around animal production units is neither technically nor economically feasible, however there are a number of management options available to minimize odor production, emission and complaints. Odors can be controlled by inhibiting anaerobic decomposition

of wastes or confining the odor generated. It can also be controlled by reducing the moisture contents, temperature, varying the pH of the waste, or applying bacteriocidal agents (Barth et al., 1984).

To overcome the odor problem many antimicrobial agents have been used to inhibit environmental urease activity. An antimicrobial agent is a chemical that kills or inhibits the growth of microorganisms. Such a substance may be either a synthetic chemical or a natural product. Antimicrobial agents affect growth in a variety of ways, and a study of the action of these agents in relation to the growth curve is of considerable aid in understanding their modes of action (Brock, 1979).

Agents that do not kill but only inhibit growth are called "static" agents. Many antimicrobial agents are effective in low concentrations, 1 to 10 parts per million. This is of practical significance since it means that the agent will be active even after it is highly diluted, making it possible to apply the agent in effective concentrations to animals or to plants without a toxicity.

To control odor, chemical methods such as using paraformaldehyde can be applied to convert ammonia to hexamethylenetetramine (Seltzer et al., 1912). Other chemical methods of controlling odors may be by counteracting or masking the odor once it is released or by inhibiting microbial activity. Some chemical methods work by inhibiting anaerobic decomposition of wastes. Another method of controlling odors is by physical confinement. Covered storage tanks and most enclosed anaerobic treatment systems are effective in

confining odors.

Recently there is a wide variety of products available to treat and prevent odors in feedlots, liquid waste storage tanks and lagoons. There are different categories of odor controlling agents. Masking agents are mixtures of aromatic oils which have a strong particular odor of their own designed to cover up the waste odor with a more desirable one. Counteractants are mixtures of aromatic oils that cancel or neutralize the waste odor so that the intensity of the mixture is less than that of the constituents. A variety of masking agents and counteractants are available for odor control. Over 38 masking agents and counteractants have been evaluated on poultry along with several digestive deodorants and chemical deodorants (Burnett et al., 1970).

Another way to control odor is by using digestive deodorants that contain bacteria or enzymes that eliminate odors through biochemical digestive processes. Also, adsorbents (products with a large surface area) may be used to adsorb odors before they are released in to the environment. Finally, chemical deodorants are strong oxidizing agents or germicides that alter or eliminate bacteria activity responsible for odor production or chemically oxidized compounds (Barth et al., 1984; Ritter 1989).

In general, masking agents and counteractant agents are the most effective, chemical deodorants are moderately effective and digestive deodorants are the least effective and must be applied frequently for effective odor control.

UREASE ACTIVITY

Ammonia is formed by bacterial degradation of urea or uric acid contained in urine and feces either excreted or stored inside animal housing area. Urease (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea to yield ammonia (NH_3)

and carbamate ($H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH$), which spontaneously hydrolyses to form carbonic acid and a second molecule of ammonia (Andrews et al., 1984). Nickel is present in the active center of urease and is involved in the catalytic activity of the enzyme.

$$H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH$$

$$H_2N\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH + H_2O \longrightarrow NH_3 + H_2CO_3$$

At physiological pH the carbonic acid dissociates and the ammonia molecules equilibrate with water to become protonated to ammonium ion, resulting in a net increase in pH.

$$H_2CO_3 \longrightarrow H^+ + HCO_3^-$$

$$2NH_3 + 2H_2O \longrightarrow 2NH_4^+ + 2OH^-$$

EFFECT OF pH ON UREASE ACTIVITY

Bacterial urease activity depends on the temperature, pH and moisture content (Burton and Beauchamp, 1986). As temperature increases, water losses increase and as water vapor is lost, the emission of objectionable compounds such as ammonia and amines increase as they distill off with water vapor (Barth et al., 1984).

Techniques for odor control are based on a number of specific principles. For example, one technique is based on chemically preventing odor release by changing the pH or by chemically destroying the responsible compounds. If the pH is above 9.5, hydrogen sulphide gas will not be released but ammonia release will be enhanced (Kreis, 1978). Low pH prevents the release of ammonia from water because most of the ammonia is present in the form of ammonium ions. Miner (1974) showed that increasing pH values increase the loss of ammonia from the liquid manure.

The pH of the waste determines the degree of ionization of its constituents and hence the vapor pressure of the constituents and their collective odor. It is known that compounds that are ionized in solution are unable to exert a vapor pressure. When this principle is applied to waste, Phillips et al., (1979) stated that hydrogen sulfide will have a considerable vapor pressure and odor when pH is near 7 but not otherwise. As pH is raised further (i.e. above 7) the acids present dissociate and the odor of the amines and ammonia become prominent.

AMMONIA MEASUREMENT

Livestock industries and odor regulatory agencies are restricted because of a lack of practical odor detection technology both qualitatively and quantitatively. Odor could be measured for intensity or quality (Barth et al., 1984). There are basic approaches to odor detection and measurement based upon the nose, wet chemistry and gas chromatography (Kreis, 1978). Olfactory evaluation is widely used for detection and evaluation of odors, but it is not selective in determining odor components. Odor and its intensity measurement is based on the number of dilutions required to reduce the concentration to a barely detectable level. Both wet chemistry and instrumental methods are used to separate, identify and quantify odor compounds. Most wet-chemical techniques suffer from lack of detection sensitivity and specificity. In contrast, gas-chromatographic (GC) techniques can be used to detect much lower ambient concentrations of odor compounds. All olfactory and analytical odor measurements have limitations in that, they are not simultaneously rapid, simple, inexpensive, and reproducible (Elliot et al., 1978).

UREA AND NITROGEN CONTENT IN URINE

Urea is a remarkably stable molecule. The half-life for the spontaneous degradation of urea is 3.6 years in aqueous solutions at 38°C (Blakeley et al., 1969).

Huge quantities of urea (NH_2-$\overset{\overset{O}{\|}}{C}$-$NH_2$) are constantly released into the environment through biological actions. For example, mammals excrete urea

as a detoxification product (Visek et al., 1972), with human urine containing 0.4 to 0.5 M urea (Griffith et al., 1987). The feces excreted by pigs contain 0.60% N, whereas urine comprised 0.40% N. Dry cows excrete 59.0% N, dairy cows excrete 62% N and poultry excretes 0.76% N (Gaur et al., 1984). Birds excrete uric acid as their primary detoxification product; however, environmental degradation of this compound also yields urea. Furthermore, urea is a well-known product of general purine catabolism (Vogels, 1976). Urea release accompanies the biodegradation of nitrogenous compounds such as arginine, and allantoin (Vogels, 1976). Urea generated by these reactions is generally short lived because of further metabolism involving the enzyme urease.

The contribution of ammonia to total nitrogen deposition and its role in the acidification of soils and surface water is now being recognized, especially in countries with intensive animal husbandry (Malanchuck and Nilsson, 1989). It is estimated that more than 90% of all ammonia production originate from agriculture and about 80% from animal production with buildings, landspreading and grazing being the predominant sources (Malanchuck and Nilsson, 1989). The management of waste concerned mainly with how these odorous materials are moved and stored, and that is influenced by the quantity and nature of bedding material used.

UREASE INHIBITORS

Urease inhibitors, (e.g. thiols, phosphate, acetohydroxamic acid, phenylphosphorodiamidate and boric acid) can be used as probes to study the enzyme mechanism. The inhibition of urease has many potential applications. For example, derivatives of hydroxamic acid have been used as chemotherapeutic agents to reduce blood ammonia levels in patients with hyperammonemia (Summerskill et al., 1967), and to reduce urine ammonia and pH in patients with urinary infections (Griffith, 1978, Munakata et al., 1980 and Rosenstein et al., 1984). Serious side effects accompany the use of these compounds (Fishbein, 1982), and they have been suggested to be teratogenic and/or carcinogenic (Rosenstein et al., 1984). Phosphoroamide derivatives (which are ~ 1000 times more potent as inhibitors) retard urinary stone growth (Millner et al., 1982) and inhibit soil urease activity (Creason et al., 1990; Martens et al., 1989 and Mulvaney et al., 1981). In addition, to the potent inhibition observed by hydroxamates and phosphoramidates, other compounds are known to inhibit urease by unknown mechanisms.

Boric Acid

Boric acid is a rapid competitive inhibitor of urease (Breitenbach and Hausinger, 1988). Boron belongs to Group III-A of the periodic table and is the only metal included among the plant micronutrient. When boron reacts with oxidizing alkaline mixtures such as sodium hydroxide (NaOH) and sodium nitrite NaO_3), it forms borates. In nature, boron is fairly rare and occurs primarily as

borates of calcium and sodium (Adriano et al., 1980).

Boric acid was found to be a simple competitive inhibitor of *Klebsiella aerogenes* urease (Ki = 0.33 mM and 11.5 mM, respectively). These values are several fold greater than those reported for *Proteus mirabilis* urease (Breitenbach and Hausinger, 1988).

Boron in Plants

Boron is indispensable for plants but not animals. It is classified as a micronutrient because such small amounts are needed by plants. Different plant species vary greatly in their boron requirements as well as in tolerating high levels in the soil. All plant parts contain boron. Boron deficiencies cause a degradation of meristematic tissue associated with a restriction in terminal growth; thickened, wilted or curled leaves; a thickened, cracked or water-soaked condition of petioles and stems, and a discoloration, cracking or rotting of fruit, tubers or roots (Robertson et al., 1981). Boron functions as a regulator in the plant metabolism of carbohydrates. Crops vary in response to boron fertilizer, with the boron utilized by crops being extremely low. Total uptake seldomly exceeds 0.45 g/acre, although an 8-ton crop of alfalfa may contain as much as 0.9 g of boron (Robertson et al., 1981). Plant analysis is one way of diagnosing both B deficiencies and toxicities.

Boron in Soil

Most of boron in soil is in tourmaline, a very insoluble mineral. The total boron in the plow layer varies greatly and is not closely related to crop

availability. Most soils range between 10 and 100 Kg B/acre, with less than 5% generally available to plants.

Plant-available boron occurs in two broad groups, mineral and organic. Mineral forms are primarily borates of calcium, magnesium and sodium. Soil micro-organisms and plants utilize such forms to produce boron-containing organic compounds. Upon death of the plants and microorganisms, decomposition is initiated, and the organic boron reverts back into a mineral form (Robertson et al., 1981).

Boron Toxicity

Animal studies indicate that there is a reasonable margin of safety between the toxic levels in animals and the levels of boron which may occur as incidental residues from the use of boron and boric acid. The toxicologic effects and levels which can be tolerated in the case of boric acid are markedly similar when doses are compared on a boron equivalent basis (Robert et al., 1972). Studies have shown that boron may be unlikely to cause a toxicosis in ruminants until a near-lethal dose is ingested (Sisk et al., 1988). Compounds containing boron are used extensively in industry, agriculture, medicine and households. Generally, the element is considered to have a low toxicity in animals. High boron concentration in liver (24 mg/Kg tissue) and in rumen contents (1,300 mg/kg rumen contents) could be toxic to cattle (Sisk et al., 1988). Clinical signs of toxicosis include weakness, staggering, depression, diarrhea and dehydration.

In man, most poisonings have been related to the use/misuse of boric acid as a bacteriostat or the accidental incorporation of boric acid into infant formulations (Lewis and Sweet, 1984; Young et al., 1949).

Human poisoning by boron compounds has been reported where large amounts were absorbed, when boric acid solutions were used for irrigation of body cavities or the gastrointestinal or urinary tracts (Goldbloom and Goldbloom, 1953). Poisoning has been reported following the absorption of boric acid when it was misused as a body powder or ointment over large areas of denuded or burned skin (Goldbloom and Goldbloom, 1953 and Valdes-Dapena and Arey, 1962). Because of its emetic properties, acute systemic toxicosis has been considered unlikely in monogastric animals. Ingested boron compounds may not elicit an emetic response in ruminants, thus increasing their vulnerability to boron toxicosis.

Compounds containing boron are used extensively in industry, agriculture, medicine, and households. The element generally is considered to have low toxicity in animals (Brockman et al., 1985). Only one accidental boron poisoning in livestock has been reported, when cattle exposed to borax (sodium borate) became ill and died at a Canadian well-drilling site.

Pine Oil

Pine oil contains high levels of terpene volatiles, including some oxygenated monoterpenes such as terpineol and limnonene (Nijholt, 1980) with known antimicrobial properties. A particularly effective pine oil appears to be

Unipine 60. The effective range of pine oil concentration in controlling ammonia production - varied from 0.001 to 50% by weight of the composition, more preferably 0.05 - 25%, and most preferably 0.1-10%, by weight of the composition (Connolly et al., 1980).

CHARACTERIZATION OF UREOLYTIC BACTERIA

The detailed characterization of any organism generally depends upon the determination of specific physiological and biochemical characteristics. The following steps are helpful in providing a framework in which identification of pure cultures can be accomplished. First, it is essential to undertake pure culture studies, since without a pure culture it is difficult to determine the characteristics of an organism that is of most general interest, such as nutritional requirements, responses to environment, metabolic products, or pathogenicity. Second, it is important to examine living cells under phase contrast and Gram-stained cells by bright-field microscopy, to determine whether the organism is Gram positive (GP) or Gram negative (GN), and to describe the morphology: rod, coccus, vibrio, spiral, etc. (Hewitt and Vincent 1989). Another way to identify the bacterium from its metabolic pattern is by using the Biolog System with either the GN MicroPlate or the GP MicroPlate. The information obtained can then be used as an entry into the keys and descriptions in Bergy's Manual of Systematic Bacteriology, with the likelihood that at least a genus name can be put on the isolate, if not a species name.

BIOLOG

Biolog MicroPlates test the ability of microorganism to utilize or oxidize a preselected panel of different carbon sources. The test yields a characteristic pattern of purple wells which constitutes a "Metabolic Fingerprint" of the capabilities of the inoculated organism. All necessary nutrients and biochemicals are prefilled and dried into 96 wells of the plate. Tetrazolium violet is used as a redox dye to colorimetrically indicate the utilization of the carbon sources. Plates start out colorless when inoculated. In wells that contain a chemical that is oxidized, there is a burst of respiration and cells reduce tetrazolium dye forming a purple color. Other wells remain colorless, as does the reference well (A-1) with no carbon source.

SHAPE AND MORPHOLOGY

Morphological properties are of major importance in the separation of many groups of bacteria. Although not exactly a morphological property, Gram-staining characteristics are generally grouped with morphology, because one can determine Gram stain and some aspects of morphology at the same time. Several of the groups of bacteria in Bergy's Manual are separated on the basis of morphology and Gram-stain.

BACTERIAL GROWTH MEASUREMENTS

Most Probable Numbers

To measure bacterial growth, precise definitions are needed. Probably the most basic definition of growth is based on the ability of individual cells to

multiply, (i.e. to initiate and complete cell division). This definition implies monitoring the increase in total number of discrete bacterial particles; which can be done either by microscopic enumeration of the particles or by electronic enumeration of the particles passing through an orifice (Gall and Curby, 1979). A second definition of growth involves determining the increase in colony forming unit (CFU). Since some cells may be dead or dying, this definition of growth may be different from the one based on the detection of discrete particles. In the long run, the increase of organisms capable of indefinite growth is the only important consideration (Kavenagh, 1963). This is the reason why colony counting and most probable number (MPN) methods are important. A third definition of growth is based on an increase in biomass. Macromolecular synthesis and increased capability for synthesis of cell components is the obvious basis for the measurement of growth by the bacterial physiologist, the biochemist, and the molecular biologist. So, cell division is an essential but minor process that seldom limits growth; what usually limits growth is the ability of enzymatic systems to rapidly utilize resources to form biomass. A final definition of growth is based upon the action of the organisms in chemically changing their environment as a consequence of the increase in biomass (Kavenagh, 1972).

The MPN method is of advantage for several reasons. First, it can be used if there is no way to culture the bacterium on solidified medium. Second, it is preferred if the growth kinetics of different cells are highly variable. If

some non selected bacteria grow rapidly and produce a large colony on solid agar, they could obscure the colonies of the selected bacteria of interest. The small colony forms may be more numerous but unmeasurable on plates because of the less numerous but highly motile or rapidly growing bacteria. Third, MPN is preferred if other bacteria not of interest are present in the sample and no selective method is available. Thus, the method has utility when the bacterium of interest produces some detectable product (e.g. colored material; antibiotic etc.). Then, even though contaminating organisms may overgrow the culture, the numbers of the bacterium in question can be estimated by the fraction of the tubes that fail to produce the characteristic product. Fourth, if agar and other solidifying materials have some factors (such as heavy metals) that may alter the reliability of the count or interfere with the object of the experimental plan, the MPN method can be used to avoid these difficulties.

Modern developments in laboratory techniques can be used to speed the execution of the MPN method. Machines are available to fill the wells of plastic trays that have as many as 144 depressions. Scanning devices designed for other purposes can be used to aid in counting the number of wells with no growth. Similarly, automatic and semiautomatic pipettes can be used to fill small test tubes

DIRECT COUNTS

Microscopic Enumeration

Microscopic enumeration in a counting chamber is a common technique

that is quick, cheap and uses equipment readily available in the bacteriological laboratory. Microscopic direct counts can also be made by membrane filter sampling and staining.

The counting chamber technique is subject to errors, but these can be overcome to a large degree by using improvements suggested by the work of Norris and Powell (1961). The major difficulty in direct microscopic enumeration is the reproducibility of filling the counting chamber with fluid.

Electronic Enumeration

The Coulter Counter (Coulter Electronics, Hialeah, Fla.) and its commercial competitors particularly the laboratory-built versions have been important in the development of bacteriology over the last 40 years. This technique is very useful in the enumeration of nonfilamentous yeasts and protozoa but not of mycelial or filamentous organisms. Although use of these counters has led to important concepts in bacteriology, the technique is difficult to apply in a valid way to estimate the volume of bacteria because of their small size and usually elongated shape (Kubitischek, 1969).

Flow Cytometry

Flow cytometry has become an extremely powerful method for the studies of many aspects of the biology eucaryotes, but the methods are only now coming into their own in the study of the biology of procaryotes, simply because the latter are smaller. The instruments, now common in hospitals and research laboratories, operates by forming a small-diameter stream of the

sample suspension. The flowing stream is examined with laser light of various frequencies and angles, and the output of the measurement circuit is used to detect when a particle passes through the light. The design permits the analysis of biomass by light-scattering methods and by staining of chemical components such as DNA. The electronic circuits allow cells to be counted very rapidly. Growth can be monitored by measuring the increase in counts in sample volume (Steen, 1990).

UREOLYTIC BACTERIA SOURCES

Odors are the most readily detected, yet probably the most difficult to resolve of the environmental pollution problems. Odors in livestock areas are a result of decomposing proteinaceous waste products such as urine, hair, feed and contaminated bedding, all at various stages of decay (O'Neill and Philips, 1991).

Ammonia is the most prevalent alkaline gas in the atmosphere, and as such plays an important role in atmospheric chemistry. In the form of ammonium aerosols ammonia can be transported over long distances, thus constituting a pollutant on an international scale.

Agricultural activities are the dominant sources of ammonia, and emissions have risen with more intensive farming and use of fertilizers. However, the direct emissions from fertilizer applications on land are generally small compared with those from livestock farming (Apsimon and Kruse-Plass, 1990).

MASTITIS

Mastitis is one of the most costly diseases of the dairy industry. Sixty to 80% of the economic losses associated with mastitis have been attributed to decreased milk yield, and the remainder results from discarded milk, veterinary costs, and animal losses (Adams et al., 1989). Despite the economic importance of mastitis hypogalactia, few studies have investigated the biological causes for the lower milk production by cows so afflicted. Common suggestions as to the causes of the hypogalactia include anorexia, bacterial toxin or inflammatory damage to mammary tissue, hormonal changes, and effects of inflammatory mediators. These effects can alter the activity of the mammary epithelium in one of three general ways: physical damage to the epithelial cells, interference with substrate availability for milk synthesis, or alteration of the metabolic activity of milk producing cells either by a reduced concentration of a galactopoietic hormone or by an increased concentration of either an inhibitory hormone or inflammatory mediator.

Mastitis, similar to most livestock diseases, is a result of the interaction between the host (cow), pathogen, and the environment. Although much research has been targeted at improvement in host immune defense and characterization of pathogen virulence factors causing mastitis, most success in control of mastitis is a result of research on utilization of management factors that reduce pathogen transmission (Smith, 1983). Contaminated bedding is believed to be one of the ways that cows become infected with mastitis.

Approximately 95% of bovine mastitis is caused by *Staphylococcus aureus, Streptococcus agalactia,* and *Streptococcus dysgalactiae* (Blosser, 1979)

The epidemiologies of clinical mastitis due to either *Escherichia coli* or *Staphylococcus aureus* are distinctly different. *E. coli* is regarded as an environmental pathogen, i.e. environmental sources are involved in the pathogenesis of this inflammation; *S. aureus* is regarded as a contagious pathogen in that the main source of intramammary infections is the udder flora.

Although *S. aureus* clinical mastitis is generally associated with high bulk milk somatic cell counts (SCC), recent studies reported clinical mastitis due to *S. aureus* in herds with a low bulk milk SCC (Hoblet et al., 1988 and Schukken et al., 1989). A survey in low bulk milk SCC in Ohio showed a low incidence of clinical *S. aureus* mastitis, but these herds were selected by criteria that included indications that *S. aureus* and *S. agalactia* had been controlled (Hogan et al., 1989). Apparently, the mechanism that decrease SCC do not necessarily eliminate *S. aureus*. The epidemiology of this microorganism in herds with a low bulk milk SCC is of particular interest as an aid in the formulation of control programs for clinical mastitis in these herds.

The most important single pathogen involved in clinical cases in herds with a low bulk SCC is *E. coli* (Erskine et al.; 1988, Hogan et al., 1989 and Schukken et al., 1989). Programs that control contagious mastitis do not control clinical mastitis caused by environmental pathogens. Hence, risk factors of clinical mastitis due to *E. coli* are of particular interest. Although few

epidemiological studies have been completed, there is evidence that the growth of microorganisms in bedding material is associated with the rate of clinical mastitis (Bramley et al., 1975 and Hogan et al., 1989). However, in herds with low colony counts in bedding material, substantial variation exists in the incidence rate of clinical mastitis. Additional herd risk factors may exist to explain this variation. Mastitis could be associated with exposure to environmental pathogens, exposure to contagious pathogens and host resistance.

Sources of Pathogens

Staphylococcus aureus is an important bacterial species implicated in mastitis. Numerous reports have suggested that *staphylococci* is ubiquitous in the cow's environment and that infections with them is inevitable. These observations were substantiated by three studies (Davidson, 1961; Newbould, 1968 and Pankey and Philpot, 1975) which emphasized that control of *staphylococcal* mastitis was dependent upon control of udder skin and intramammary and udder infections. *Staphylococci* do not persist on healthy teat skin but readily colonize teat canals if a lesion is near the teat apex. Organisms multiplying in infected lesions or colonized teat canals are situated ideally for transfer into the udder.

Streptococcus agalactia is another pathogen implicated in mastitis The only significant reservoir for *S. agalactia* is infected udders. Although the organism may be isolated from bedding, milking equipment, and other objects,

its presence is a consequence of recent contamination with infected milk. In absence of intramammary infection (IMI) the organism will disappear from secondary sites.

Other pathogens such as *coliforms* and *pseudomonads,* are the most important secondary pathogens in most herds. Incidence of IMI is generally low, although outbreaks may occur when conditions develop that greatly increase exposure to the organisms. *Coliforms* are found in manure and bedding. P*seudomonas* mastitis may originate from contaminated water supplies, soil, or inadequately cleaned milking machines. Mastitis associated with exposure to environmental pathogens such as *E. coli* could be due to cubicle cleaning, rubber mats in calving area, and udder preparation with water. Whereas, in *S. aureus* mastitis could result due to the source of drinking water.

Mastitis Control

Hygiene and therapy are two important components to control mastitis. Conscientious application of these practices significantly reduces intramammary infection, especially when they are applied in concert with superior management. These two components operate independently, and response is maximum when both are applied. Hygiene acts by reducing the frequency of infection and are of value in reducing the incidence of new infections. So, hygiene must prevent or at least reduce, transmission of pathogens from one teat to another on the same cow as well as between cows. If transmission from udder to udder could be prevented or reduced substantially,

there would be a concomitant decrease incidence of new intramammary infection (IMI). The most effective hygiene and therapy practices are dipping of teats after milking and treating each quarter with antibiotics at the end of lactation (Philpot and Pankey, 1975). To decrease the incidence of mastitis, good milking hygiene procedures should be followed, as well as keeping the bedding dry and clean to decrease the presence of pathogenic bacteria (Philpot and Pankey, 1975).

In summary, odors are the most readily detected, yet difficult to solve, environmental pollution problem for different species. Livestock odors are generally regarded as nuisance pollutants. Ammonia production from livestock buildings are caused by the anaerobic decomposition of microorganisms. Urease catalyzes the hydrolysis of urea yielding ammonia and carbamate, which spontaneously hydrolyzes to form carbonic acid and a second molecule of ammonia. Odors could be suppressed by lowering the temperature, moisture, pH-value, and following general hygienic rules. Also, applying odor control agents such as chemicals, masking agents, and antimicrobial agents are another way to decrease ammonia. Boric acid and pine oil could be used as urease inhibitors, thus inhibiting ammonia production. In addition, bedding can decrease ammonia release if sufficiently used, (e.g. when chopped straw is used as a bedding). It has been reported that this mixture has a more acceptable odor than the manure (Williams et al., 1981). This may be due to the physical structure of straw encouraging compositing (i.e. thermophilic

aerobic decomposition), thereby inhibiting anaerobic decomposition and thus the production of more malodorous compounds.

RESEARCH OBJECTIVES

The goal of this research was to determine the inhibitory effects of boric acid, pine oil and their combination on ammonia production in different bedding materials contaminated with livestock urine or chicken manure and on the growth of pure cultures of ureolytic and mastitis causing bacteria.

SPECIFIC OBJECTIVES

1- To determine the ammonia production emanating from bedding substrates contaminated with urine from different livestock.

2- To determine the inhibitory effects of boric acid, pine oil and their combination on ammonia production in different livestock bedding materials contaminated with livestock urine.

3- To determine the inhibitory effect of boric acid, pine oil and their combination on the growth of pure cultures of ureolytic bacteria isolated from contaminated livestock beddings.

4- To characterize the ureolytic bacteria isolated from bedding materials contaminated with livestock urine.

5- To determine the inhibitory effects of boric acid, pine oil and their combination on the growth of pure cultures of mastitis causing organisms.

MATERIAL AND METHODS

AMMONIA SAMPLING TECHNIQUE:

Kitagawa tubes (Alltech Associates, Inc. 2051 Waukegan Road, Deerfield, IL 60015) were used to measure ammonia production (ppm) in this study. To validate the technique system a stock of aqueous solution of 1 M urea was prepared and used to prepare 0.5 M and 0.25 M concentration. Fresh cow urine (collected during urination) was also used. In 9 oz wide mouth mason jars, 50 ml of 0.5 M or 0.25 M urea and either 2X or 4X diluted cow urine was thoroughly mixed with 50 g sawdust. A piece of parafilm was placed loosely on top of each jar, which was then incubated at room temperature (24°C) for 5 days. All jars were prepared in duplicates. Ammonia production was measured at 24 hours interval for three consecutive days, by drawing 60 cc air from the jars head space (approximately 5 cm over the urine or urea contaminated bedding) using the Kitagawa tubes.

To carry out the sampling process, the ends of the tube were first removed using a tip cutter. The end of the gas detector tube was inserted in a small rubber tube connector, and the rubber tube was then connected to a 60 ml glass syringe. The syringe was used to ensure that the gas volume withdrawn from all jars was exactly the same. Each jar lid was perforated to allow entry of the Kitagawa tubes and air sampling to take place. An arrow printed on the tubes indicated the direction of ammonia flow. These tubes use

a highly purified and reactive chemical (phosphoric acid) as the detecting reagent which is adsorbed on a purified silica sand, silica gel or activated alumina. Upon exposure to ammonia a distinct color change (from purple to pink) occurs in the tube giving a semi-quantitative indication of ammonia concentration. Each tube has a calibrated scale to allow a direct and precise reading.

UREOLYTIC BACTERIA SOURCES

Three sets of jars were prepared. In each set, two of the three possible ureolytic bacteria sources (urine, bedding and environment) were eliminated by autoclaving. To investigate the environment role as a source of ureolytic bacteria, both autoclaved sawdust, jars and filter sterilized urea solution were used (Set 1). Sawdust and 9 oz empty clean jars were autoclaved (20 minutes, 110 °C, at 80 psi), also urea was filtered (using 4 mm whatman paper) under gravity. While autoclaved jars, filter sterilized urea and regular bedding (not autoclaved) were used to determine the bedding contribution (Set 2). Finally, to investigate if urine was a source of ureolytic bacteria (Set 3), freshly collected cow urine was used with both autoclaved jars and bedding (sawdust). In all the 3 sets, 50 g of sawdust and 50 ml of urea (0.25 or 0.5 M) or urine (50 or 25 ml) were used. All sets were prepared in triplicates.

A piece of parafilm was placed loosely on top of each jar and the jars incubated at room temperature (24°C) for 4 days. Starting at day 5 ammonia production was measured for 7 consecutive days using Kitagawa tubes (as

previously described on page 28).

EFFECT OF pH ON AMMONIA PRODUCTION

To determine the effect of pH on ammonia production, change in pH relative to ammonia production was measured for 3 consecutive days. Treatments consisted of 5 jars: a negative control, a positive control and either, pine oil (PO), boric acid (B) or their combination (M). Positive control (C1) was prepared by adding H_2O (50 ml) + urine (50 ml). Control (C) H_2O (48.05 ml) + triton (0.2 ml) + neodol (0.4 ml) + NaOH (1.35 g); pine oil (PO) H_2O (44.70 ml) + NaOH (1.35 g) + triton (0.2 ml) + neodol (0.4 ml) + PO(3.35 ml); boric acid (B) H_2O (39.72 ml) + NaOH (1.35 g) + B(8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) and; combination (M) H_2O (36.37 ml) + NaOH (1.35 g) + boric acid (8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) + pine oil(3.35 ml). The final concentration of each treatment was, 16.8 % for boric acid, 6.7% for pine oil and 16.8% and 6.7% for the combination.

An amount of 1.5 ml of each treatment was added to 50 ml sawdust and then contaminated with 50 g fresh cow urine. Two sets of jars were used (Set 1) to measure ammonia production and (Set 2) to measure pH changes. To measure ammonia production, a piece of parafilm was placed loosely on top of each jar. All jars were incubated at room temperature (24°C) for five days. To measure pH changes, 10 g of each jar content in (Set 2) was recovered and washed with 10 ml of water and pH values were measured using a pH electrode (Orion 910500). Ammonia production and pH values were measured

for three consecutive days.

AMMONIA PRODUCTION FROM BEDDING MATERIAL CONTAMINATED WITH DIFFERENT LIVESTOCK URINE

Fresh urine was collected during urination from three different cows, three pigs, and three horses in sterile containers and used directly. Fresh chicken manure (manure that had accumulated overnight in a clean tray) were pooled. Animals were randomly chosen from Michigan State University livestock farms. Three replicate jars were prepared for each animal and bedding (sawdust).

Fifty ml of urine or 50 g chicken manure were mixed with 50 g sawdust, in 9 oz clear wide mouth glass jars. Fifty ml of distilled water was used as the control. Samples were thoroughly mixed using a spatula to ensure homogeneity, and equal distribution. Jars were loosely covered with a piece of parafilm and held at room temperature (24°C). Ammonia emission was measured in the head space of each jar. Precautions such as: keeping the jars unmoved, and unshaken, no air draft, normal room temperature were taken to incubate the jars under stable and undisturbed conditions. Ammonia concentration (ppm) was measured once every 24 hours for 7 consecutive days using Kitagawa tubes.

STATISTICAL ANALYSIS

The statistical design was a complete randomized design, one way analysis of variance according to the model shown in (I). This model was applied to study the variations in ammonia production among animals waste

(cow, horse, pig urine and chicken manure).

(I) $Y_{ij} = \mu + \alpha_j + E_{ij}$

Where:

Y_{ij} = Ammonia production from one species.

μ = General mean.

α = Species effect.

E = Residual error.

i = Readings within species (21 reading).

j = Animal index (4 species).

Ammonia production in different days (day 1 to 7) was analyzed for each species separately by applying a completely randomized design, one way analysis of variance according to model in (II).

(II) $Y_{ij} = \mu + \beta_j + E_{ij}$

Where:

Y_{ij} = Ammonia production from one species.

μ = General mean.

β = Days effect.

E = Residual error.

i = Readings within days (3 readings).

j = Days index (7 days).

THE INHIBITORY EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON AMMONIA PRODUCTION FROM DIFFERENT BEDDING MATERIALS CONTAMINATED WITH LIVESTOCK URINE

Fifty ml fresh urine (collected during urination) from three cows, three pigs, three horses and 50 g of fresh pooled chicken manure (e.g. manure that had accumulated overnight in a clean tray) were collected and used. Different bedding materials (e.g. sand, sawdust, straw, corncobs, newspaper and woodshaving) were obtained from Michigan State University barns. Fifty gram of each bedding and 300 g of sand were mixed with either 50 ml urine or 50 g manure. All bedding materials used in this study represent the commonly used beddings for each livestock species. Cattle urine was examined with sawdust, chopped straw, woodshaving, shredded newspaper, ground corncobs and sand. Horse urine was examined with sawdust, chopped straw, woodshaving, and sand. Pig urine was examined with sawdust, sand, and woodshaving and poultry manure was examined with sawdust and woodshaving. Four treatments, consisting of: Control (C) H_2O (48.05 ml) + triton (0.2 ml) + neodol (0.4 ml) + NaOH (1.35 g); pine oil (PO) H_2O (44.70 ml) + NaOH (1.35 g) + triton (0.2 ml) + neodol (0.4 ml) + PO(3.35 ml); boric acid (B) H_2O (39.72 ml) + NaOH (1.35 g) + B(8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) and combination (M) H_2O (36.37 ml) + NaOH (1.35 g) + boric acid (8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) + pine oil(3.35 ml). All treatments were prepared to a final volume of 50 ml. Therefore, the final concentration of each treatment was, 16.8 % for boric acid, 6.7% for pine oil and 16.8% and 6.7%

for the combination.

Because of the marked differences in the mass of the different beddings relative to their weight (e.g. sand vs newspaper) bulky bedding material such as, newspaper, and straw had to be cut into smaller pieces than normally used. Larger jars (32 oz capacity) were used for newspaper, woodshaving and straw; while 9 oz jars were used for sand and corncobs, and 16 oz for sawdust. All treatments were done in triplicate. An amount of 1.5 ml of each treatment (C, B, PO and M) was added to the bedding (50 g)-urine (50 ml) mixture and mixed thoroughly using a spatula. Treatments were applied to newspaper, straw and woodshaving using a trigger-sprayer bottle while mixing was going on. This was done to ensure equal distribution of urine and treatment and to overcome the fast absorbing characteristic of these beddings. A piece of parafilm was loosely placed on top of each jar and jars were held at room temperature (24°C) for five days. Ammonia production (ppm) was measured at 24 hr intervals for seven consecutive days, using Kitagawa tubes.

STATISTICAL ANALYSIS

Ammonia production was analyzed for all species and treatments by a three way analysis of anova, completely randomized design according to model (I).

(I) $$Y_{ijkl} = \mu + \alpha_i + D_j + \gamma_l + \alpha D_{jk} + \alpha\gamma_{jl} + D\gamma_{kl} + \alpha D\gamma_{jkl} + E_{ijkl}$$

Where:

Y_{ijkl} = Ammonia production.

μ = General mean.

α = Species effect.

D = Treatment effect.

γ = Bedding effect.

αD = Interaction of treatment and species.

$\alpha\gamma$ = Interaction of species and bedding.

Dγ = Interaction of treatment and bedding.

αDγ = Interaction of species, treatment and bedding.

E_{ijkl} = Residual error.

i = readings with treatment, animal, and bedding (3 readings).

j = treatment index (1,2,3,4).

K = species index (1,2,3,4).

L = bedding index.

Treatment effect within each species and bedding was analyzed by one way analysis of variance according to model (II).

(II) $Y_{ij} = \mu + D_j + E_{ij}$

where:

Y = Ammonia production.

μ = General mean.

D = Treatment effect.

i = Observation index.

j = Treatments index (4 treatments).

The data was analyzed by a complete randomized design, one way analysis of variance according to model (III). This model was applied to study the variations in ammonia production among animals waste (cow, horse and pig urine and chicken manure).

(III) $Y_{ij} = \mu + \alpha_j + E_{ij}$

Where:

Y_{ij} = Ammonia production from one species.

μ = General mean.

α = Species effect.

E = Residual error.

i = Readings within species (21 reading).

j = Animal index (4 species).

To study the effect of days on ammonia production the data was analyzed by a complete randomized design, one way analysis of variance according to model (IV).

(IV) $Y_{ij} = \mu + \beta_j + E_{ij}$

Where:

Y_{ij} = Ammonia production.

μ = General mean.

β = Days effect.

E = Residual error.

i = Readings within day (12 readings).

j = Days index (7 days).

THE INHIBITORY EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON THE GROWTH OF PURE CULTURES OF UREOLYTIC BACTERIA ISOLATED FROM CONTAMINATED LIVESTOCK BEDDINGS

The direct effect of the treatments (C, PO, B, and M) on ureolytic bacteria was studied using the inhibition zone method (Petersdorf and Sherris, 1965). Twenty-five ml of molten 2% agar, 25 ml of 8% nutrient broth and 5% glucose held at 45 °C were mixed with 4 ml pure ureolytic bacteria culture and carefully poured over a solidified lower agar layer (25 ml of 2% agar). To assure similarity of bacterial numbers in samples, the inoculum (ureolytic bacteria) was grown in nutrient broth supplemented with glucose to OD_{600} value of 0.4. Using a cork boring device (size 6) (Ray and Mark, 1992) a well for each treatment (C, PO, B, M) was carefully cut into the top agar layer of each plate. Each well was then filled with 300 ul of each treatment, and the plates were incubated at 37°C for 48 hours. The clearing zone of inhibition in each well was determined by measuring the distance between the zone edge and the well edge. Treatments were prepared as follows: Control (C)- H_2O (48.05 ml) + triton (0.2 ml) + neodol (0.4 ml) + NaOH (1.35 g); Pine oil (PO)- H_2O (44.70 ml) + NaOH (1.35 g) + triton (0.2 ml) + neodol (0.4 ml) + PO(3.35 ml); boric acid (B) H_2O (39.72 ml) + NaOH (1.35 g) + B(8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) and Combination (M) H_2O (36.37 ml) + NaOH (1.35 g) + boric acid (8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) + pine oil(3.35 ml). All treatments were prepared to a final volume of 50 ml. Therefore, the final

concentration of each treatment was, 16.8 % for boric acid, 6.7% for pine oil, and 16.8% boric acid plus pine oil 6.7% for the combination, with the exception of omitting NaOH from all the treatments and substituting its exact amount with distilled water. This step was necessary to avoid the alkaline effect of NaOH on pH which could affect bacterial growth.

Isolation Of Pure Cultures Of Ureolytic Bacteria

To isolate pure cultures of ureolytic bacteria, fifty ml fresh urine (collected during urination) of three species (dairy cows, horses, and pigs) as well as 50 g chicken manure (fresh manure accumulated overnight in a clean tray) were mixed thoroughly using a spatula with 50g of different beddings (sawdust, chopped straw, sand, corncobs, shredded newspaper, and woodshaving). Mixtures were placed in sterile glass jars, covered with a piece of parafilm and held at room temperature (24°C) for five days, until ammonia concentration reached their peak levels. 900 ml distilled water was then added to each contaminated bedding-urine mixture (100 g) in the jars. Mixtures were transferred to sterile ziplock bags and agitated for 2 minutes in a Stomacher (Tekmar catalog # 3500). Serial dilutions were prepared by adding 0.1 ml of the bedding-urine mixture to 9.9 ml (10^{-1}) peptone. This procedure was carried out to 10^{-9} dilution and the diluted tubes were incubated at 37°C overnight. Sterile solidified agar (2%), urea broth (3.8 %) plates were inoculated with 0.2 ml of each dilution and incubated overnight at 37°C. As an indication of the presence of ureolytic bacteria, urea agar plates changed color from amber to

pink. Ammonia production resulted in a pH increase causing the plates to change color due to the phenol red indicator dye in urea broth. To isolate ureolytic bacteria, 4 ml of filter sterilized urea broth was inoculated with individual bacterial colonies picked from positive plates and incubated overnight at 37°C. Tubes containing positive ureolytic bacteria changed the color of the broth medium from red to pink.

CHARACTERIZATION OF UREOLYTIC BACTERIA ISOLATED FROM BEDDING MATERIALS CONTAMINATED WITH LIVESTOCK URINE

Gram staining was performed on all pure cultures of bacteria isolated from different bedding materials contaminated with livestock urine. The Biolog Classification System was used to characterize the ureolytic bacteria isolated. Isolates were freshly grown on sterile Biolog Universal Growth Medium (BUGM) (3.7%) and Becto agar (1.5%), pH 7.3-7.4. Fresh pure cultures were used for analysis by Biolog since bacteria can lose their viability and metabolic activity in stationary phase. Pure cultures of bacteria were successfully isolated from chopped straw, sand, sawdust, and ground corncobs contaminated with cow urine; from sand, woodshaving, chopped straw and sawdust contaminated with horse urine; from woodshaving, sand, and sawdust contaminated with pig urine; and from woodshaving and sawdust contaminated with chicken manure.

Cells were isolated from the agar plates with a sterile swab so as not to carry any nutrients from the agar medium into the suspension. To assure equality in the inoculum, the optical density of cultures were determined for all inocula. The turbidimeter was blanked (transmittance = 100%) with a control

tube containing uninoculated saline (9% NaCl). The necessary turbidity range was between 57% and 60%. The inoculum density was adjusted to the required turbidity range by adding more saline solution or by adding more cells.

Microlog 3 computer software (Release 3.5) was used to interpret the readings according to the Biolog procedure. MicroPlate lids were removed and the MicroPlates were placed into the drawer of the reader. The program was set at a wavelength 630 nm. Readings of the MicroPLates were taken after 4, 24 and 48 hr of incubation. After 4 hr of incubation, the adequate match index must be 75% to be considered an acceptable species identification and 50% after 16-20 hr of incubation. These two threshold values give comparable levels of accuracy.

All wells of the MicroPlates were filled precisely with 150 μl of the bacterial suspension using 8-channel repeating pipetter. The MicroPlates were covered with their lids, and incubated at 35°C for either 4 hr or overnight to allow the pattern to form. The pattern of positive purple wells was then keyed into Biolog's MicroLog computer program which automatically cross-referenced the produced pattern to an extensive library of known bacterial species in the database. If an adequate match was found, an identification of the isolate is made.

Most Probable Numbers

To estimate the number of ureolytic bacteria present in urine and bedding materials contaminated with livestock urine from either pig, horse, cow or

poultry manure, six replicates of serially diluted (10^{-2} to 10^{-10}) urine contaminated bedding material (e.g. chopped straw, woodshaving, sawdust, ground corncob, shredded newspaper, and sand) were prepared. Briefly, to prepare the 10^{-2} dilution, 1ml of urine contaminated with bedding material was added to 9ml of urea broth. Then 1 ml of the 10^{-2} dilution was added to 9ml urea broth to prepare the 10^{-3} dilution. This procedure was carried over up to (10^{10}). Also, 6 replicates of fresh urine and pooled manure were also prepared. Urea broth (38.7 g/l) was used as diluent for the serial dilutions.

Fifty ml fresh urine of each animal was poured over 50 g of each bedding material that was placed in cheese cloth. With poultry manure, 50 g manure was mixed with each bedding, placed in cheese cloth and then washed with 50 ml distilled water. 1.0 ml of each filtrate was added to 9 ml urea broth (10^{-1}). Serial dilution tubes (10^{-2} to 10^{-10}) were incubated at 37°C for two days. Tubes containing ureolytic bacteria changed the color of the broth medium from light red to pink and the most probable numbers was determined from the multiple tubes (Benson, 1973).

THE INHIBITORY EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON THE GROWTH OF PURE CULTURES OF MASTITIS CAUSING ORGANISM

Pure cultures of *Streptococcus agalactia, Staphylococcus aureus and E. coli* were obtained from Michigan State University Veterinary School, Diagnostic Laboratory. The strains were grown on 3.8% Mueller Hinton II agar. Mueller-Hinton is a good compromise for routine susceptibility testing because

it shows good batch-to-batch reproducibility, enables most pathogens to grow satisfactory, and is low in tetracycline, trimethoprim, and sulfonamide inhibitors (Balows, 1974 and Acar, 1980). These drugs act against bacteria by preventing the synthesis of para-aminobenzoic acid's (PABA) through competitive inhibition. Most media contain an abundance of the main PABA derivatives, folic acid, and observable inhibition around sulfonamide discs is therefore prevented by satisfying bacterial needs directly. Mueller-Hinton contains traces of this antagonist (Petersdorf, 1963; Ryan, 1970 and Acar, 1980). A colony from each bacterial strain was suspended in 10 ml of 8% nutrient broth and 5% glucose. Tubes were incubated at 37°C overnight until a suspension of moderate cloudiness developed. Twenty-five ml Mueller-Hinton agar (3.7%) and 25 ml of the nutrient broth (8%) and glucose (5%) were inoculated with 4 ml of the strain cultures and poured into sterile 15 cm Petri plates (Bauer, 1964) to a depth of 4 mm on a level surface. The plates were allowed to dry for 15 minutes (Lennette, 1980; Acar, 1980 and Bauer, 1964). Half-inch paper discs impregnated with the treatment were deposited on the plate. To insure full contact of the discs with the agar surface the discs were pressed flat against the agar by applying firm but gentle pressure using an alcohol-flamed forceps (Finegold and Martin, 1982; Balows, 1974, Lennette, 1980 and Bauer, 1964). In addition, this prevents discs from falling off when the plate is inverted for incubation. Plates were incubated at 37°C for overnight or until a good growth is obtained. The discs were no closer than 1.5

cm to the edge of the plate, and rested about 3.0 cm apart from each other, to avoid overlapping zones and thus inaccurate readings.

Two sets of treatments were prepared, in which NaOH was omitted from one set and substituted by its exact amount of sterile water (all treatments were prepared as follows: Control (C)- H_2O (48.05 ml) + triton (0.2 ml) + neodol (0.4 ml) + NaOH (1.35 g); pine oil (PO)- H_2O (44.70 ml) + NaOH (1.35 g) + triton (0.2 ml) + neodol (0.4 ml) + PO(3.35 ml); boric acid (B)- H_2O (39.72 ml) + NaOH (1.35 g) + B(8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) and combination (M)- H_2O (36.37 ml) + NaOH (1.35 g) + boric acid (8.34 ml) + triton (0.2 ml) + neodol(0.4 ml) + pine oil(3.35 ml). All treatments were prepared to a final volume of 50 ml. Therefore, the final concentration of boric acid and/or pine oil in each treatment was, 16.8 % for boric acid, 6.7% for pine oil and 16.8% boric acid and 6.7% pine oil for the combination.

RESULTS AND DISCUSSION

VALIDITY OF AMMONIA MEASUREMENT

Odorants associated with animal production exist at low levels, therefore concentrating procedures are often needed for odor measurement. The major problem in assessing odor nuisance is that simple technology is not available for quantitatively detecting and measuring odors. Since the obstacle to regulating and managing odors is the lack of quick, unbiased, and reproducible methods, future research on economic odor measurement methods should be greatly encouraged. Elliott et al. (1978) discussed the analytical methods for detecting odors and concluded that quick and economical means of measuring odors from waste management systems are needed in order to develop odor control technology.

There are basic approaches to odor detection and measurement based upon the nose, wet chemistry and gas chromatography (Kreis, 1978). Olfactory evaluation is widely used for detection and evaluation of odors, but it is not selective in determining odor components. Odor and its intensity measurement is based on the number of dilutions required to reduce the odor concentration to a barely detectable level. Both wet chemical and instrumental methods are used to separate, identify and quantify odor compounds. Most wet chemical techniques suffer from lack of detection sensitivity and specificity. In contrast, gas-chromatographic (GC) techniques can be used to detect much-lower ambient concentrations of odor compounds. All olfactory

and analytical odor measurements have limitations, they are not simultaneously rapid, simple, inexpensive, and reproducible (Elliot et al., 1978).

To validate the ammonia measuring system, Kitagawa tubes were used to measure ammonia production from sawdust contaminated with cow urine or urea. When cow urine was diluted 4X with water, ammonia production averaged 50 ppm, and reached 120 ppm in the jars containing sawdust contaminated with 2X diluted urine. Furthermore, ammonia production from jars containing the 0.25 M urea was 120-220 ppm and reached 230-430 ppm in the jars containing 0.5 M urea (Table 1). In the third day ammonia production from the urea contaminated bedding decreased, this decrease might be due to depletion of urea source. Howevwer in the urine set the urea source was still adequate to produce constant amount of urea.

These results indicate that ammonia concentration almost doubled (95 and 98 %) when either urea or urine concentrations were doubled respectively. Thus, using the Kitagawa tubes appeared to be an efficient, practical and relatively accurate means of measuring ammonia production.

Table 1: Validity Of Ammonia Production Measuring System

	Urea		Urine	
Day	0.25M	0.5M	25ml	50ml
	Head Space Ammonia Production in ppmb[a]			
2	220	430	50	120
3	150	300	60	120
4	120	230	50	120

[a] Each value represents the mean of two readings. Ammonia production was measured at 24 h interval using Kitagawa tubes.

UREOLYTIC BACTERIA SOURCES

Environment (atmosphere, contamination...etc), bedding and urine are the three possible sources of ureolytic bacteria. To investigate the contribution of each source to ureolytic bacteria contents, 3 sets of jars were prepared. To examine environmental contribution, autoclaved sawdust, jars, and filter sterilized urea solution were used in Set 1. To examine bedding contribution, autoclaved jars, filter sterilized urea solution and regular bedding (not autoclaved) were used in Set 2. Finally, to examine urine contribution freshly collected cow urine was used with both autoclaved jars and bedding (sawdust) in Set 3. In all 3 Sets, 50 g of sawdust and 50 ml of urea (0.25 or 0.5 M) or urine (25 or 50 ml) were used.

Ammonia production was high at day one of measurement (after 4 days of incubation) for urine (0 ppm for 2X diluted urine and 100 ppm for nondiluted urine) and bedding (200 ppm for 0.25 M urea and 390 ppm for 0.5 M urea) in Set 3 and Set 2 respectively. Whereas, ammonia production was not detectable until the second day of measurement (after 5 days of incubation) in Set 1. In general, ammonia production from Set 1 was low (50 ppm) compared to Set 2 and 3 (Table 2). All readings of samples containing 0.5 M urea were higher than those containing 0.25 M urea. These results suggest that bedding and urine are the main sources of ureolytic bacteria; and the environment plays a minor role in terms of supplying ureolytic bacteria.

It appeared that sawdust is a major source of ureolytic bacteria (Set 2),

Table 2: Source Of Ureolytic Bacteria Responsible For Ammonia Production In Urine Contaminated Bedding

DAY	SET 1		SET 2		SET 3	
	Urea				Urine	
	0.25M	0.5M	0.25M	0.5M	25ml	50ml
	Head Space Ammonia Production in ppm[a]					
1	0	0	200	390	0	100
2	0	0	220	430	50	120
3	0	0	150	500	60	120
4	0	0	120	530	50	120
5	0	0	180	340	80	200
6	50	50	150	250	110	200
7	130	100	250	470	40	110

[a] Each value represents the mean of three readings. Ammonia production was measured at 24 h interval using Kitagawa tubes.

To examine environmental contribution, autoclaved sawdust, jars, and filter sterilized urea solution were used in Set 1. To examine bedding contribution, autoclaved jars, filter sterilized urea solution and regular bedding (not autoclaved) were used in Set 2. Finally, to examine urine contribution freshly collected cow urine was used with both autoclaved jars and bedding (sawdust) in Set 3.

since ammonia production was 200 and 390 ppm on day 1 and reached 150 and 500 ppm in day 3 (after incubation). It has been reported that organic bedding materials such as sawdust, woodshaving, straw, newspaper and corn cobs contain higher levels of bacteria compared to inorganic bedding materials such as sand (Hogan et al., 1988)

The observed decline in ammonia production from Set 2 at day 4 when 0.5 M urea was used might have been due to urea depletion or a decrease in the ureolytic bacteria numbers. Ammonia production was fairly constant in Set 3 (urine) and was much less than the ammonia production detected in Set 2 (bedding), which suggest that the presence of ureolytic bacteria in urine was not as high as in bedding. In conclusion, these results indicate that bedding material is probably the main source of ureolytic bacteria followed by urine and environment in a descending order. Studies of the ureolytic bacteria content in different bedding materials would be useful in determining the best bedding to be used as livestock buildings.

pH EFFECT ON AMMONIA PRODUCTION

A considerable proportion of nitrogen in urine is in the form of urea from which ammonia is readily released by the enzymatic activity of urease. Urine and feces characteristics differ among species and as a result ammonia production from different urine materials would vary. In addition, the amount of excreta differ among species which affects the contribution of each species in ammonia production; for example, the nitrogen average in dry cow urine is 59.0%. Feces consist of about 75% to 85% moisture, 15% organic matter and 2 to 5% mineral matter (Neelakantan and Singh, 1975). Feces excreted by hogs and pigs contained 0.60% N, whereas, urine comprised 0.40% N. The average excretion of hog manure is about 6.8% of the body weight/hog/day. Poultry excreta is a rich organic manure, since liquid and solid are excreted together resulting in no urinary loss. Poultry manure degrades very quickly. If left exposed, it may lose up to 50% of its nitrogen within 30 days. The excreta from hens that are 1 to 6 months old ranged from 229 to 888 g/head/day depending on their age (Morino, 1975). The poultry excreted about 5% of their body weight/day. Chicken up to 13-weeks of age excrete about 274g/head/day. Fresh poultry excreta comprised about 80% moisture, and 0.76% N.

Urea is not stable in the environment, it is rapidly hydrolyzed to ammonia and carbamate through the enzymatic reaction of urease (Blakeley et al., 1969). Ammonia is formed by bacterial degradation of urea or uric acid from urine and

feces which are just excreted or stored inside the animal house. Bacterial activity depends on temperature and pH. The influence of temperature is demonstrated by seasonal fluctuations of the ammonia-N loss in relation to the aerial temperature in animal barn (Burton and Beauchamp, 1986). The lower the temperature the lower the activity of the bacteria (Hartung, 1991) and consequently the lower the ammonia production.

Adjustment of pH slows microbial activity and can be used to limit the production of the primary odorants. High pH will limit the emission of volatile acids and hydrogen sulphide. However, low pH will diminish release of ammonia and amines. As pH is raised the acids present in manure will dissociate and odors of ammonia become prominent (O'Neill and Phillips, 1991). Therefore, low pH-values prevent the release of ammonia from urine because most of the ammonia is present in the form of ammonium ion. Miner (1974) showed that increasing pH-values increases the loss of ammonia from liquid manure. In theory, pH control could be used to reduce odorants production, but it is expensive to decrease urinary or waste pH. In addition this treatment may render the waste undesirable for utilization. Also, pH control to reduce emission of one or more odorants encourages the release of other odorants which could end up to be a self-defeating practice (Barth et al., 1984).

Since the designed treatments included triton, neodol and sodium hydroxide, the negative control (C) was the combination of these chemicals (slurry). On the other hand, the positive control (C1) did not contain these

additives. All jars were incubated at room temperature for five days. The results (Table 3) indicated that the slurry (C) did not reduce ammonia production at least for the first two days after the incubation period. On day 3 (after incubation) ammonia production from C (negative control) was less than C1 (positive control) (Table 3). This difference might be due to the presence of triton, neodol and sodium hydroxide in control. Therefore, C will be used as the control in the body of this thesis to be compared against the other treatments. All three treatments showed a reduction in ammonia production relative to C. Maximum pH and ammonia production were achieved at day four (pH >9.5). If pH is above 9.5, hydrogen sulphide gas will not be released but ammonia release will be enhanced (Seltzer et al., 1969). The increase in pH could be attributed to the ammonia liberated by the decomposing substrate (Nodar et al., 1990).

The increase in pH was marginal in day two and three. On the fourth day pH showed a dramatic increase accompanied by increase in ammonia production. However, the treated jars still show less ammonia production relative to the control. Interestingly, ammonia production during the three days was different among the treatments. However, pH level was almost the same. This suggests that the treatments do not affect ammonia production by changing the pH.

Table 3: Ammonia Production And pH Values From Sawdust Contaminated With Cow Urine.

TRT.[a]	DAY (2)		DAY (3)		DAY (4)	
	NH_3	pH	NH_3	pH	NH_3	pH
	(ppm)		(ppm)		(ppm)	
[a]C1	200	9.2	360	9.3	650	10.8
[b]C	150	9.5	330	9.3	490	10.8
[c]B	100	9.0	200	9.3	300	10.3
[d]PO	100	9.2	280	9.3	390	10.3
[e]M	110	9.0	250	9.2	370	10.0

The final concentration of each treatment was, 16.8 % B in boric acid treatment, 6.7% PO in pine oil treatment and 16.8% B and 6.7% PO for the combination.

[a]C1 = Positive control (urine and bedding only), [b]C = Control (neodol, triton or sodium hydroxide plus urine and bedding).

[c]B = Boric acid, [d]PO = Pine oil, [e]M = Pine oil and boric acid combination.

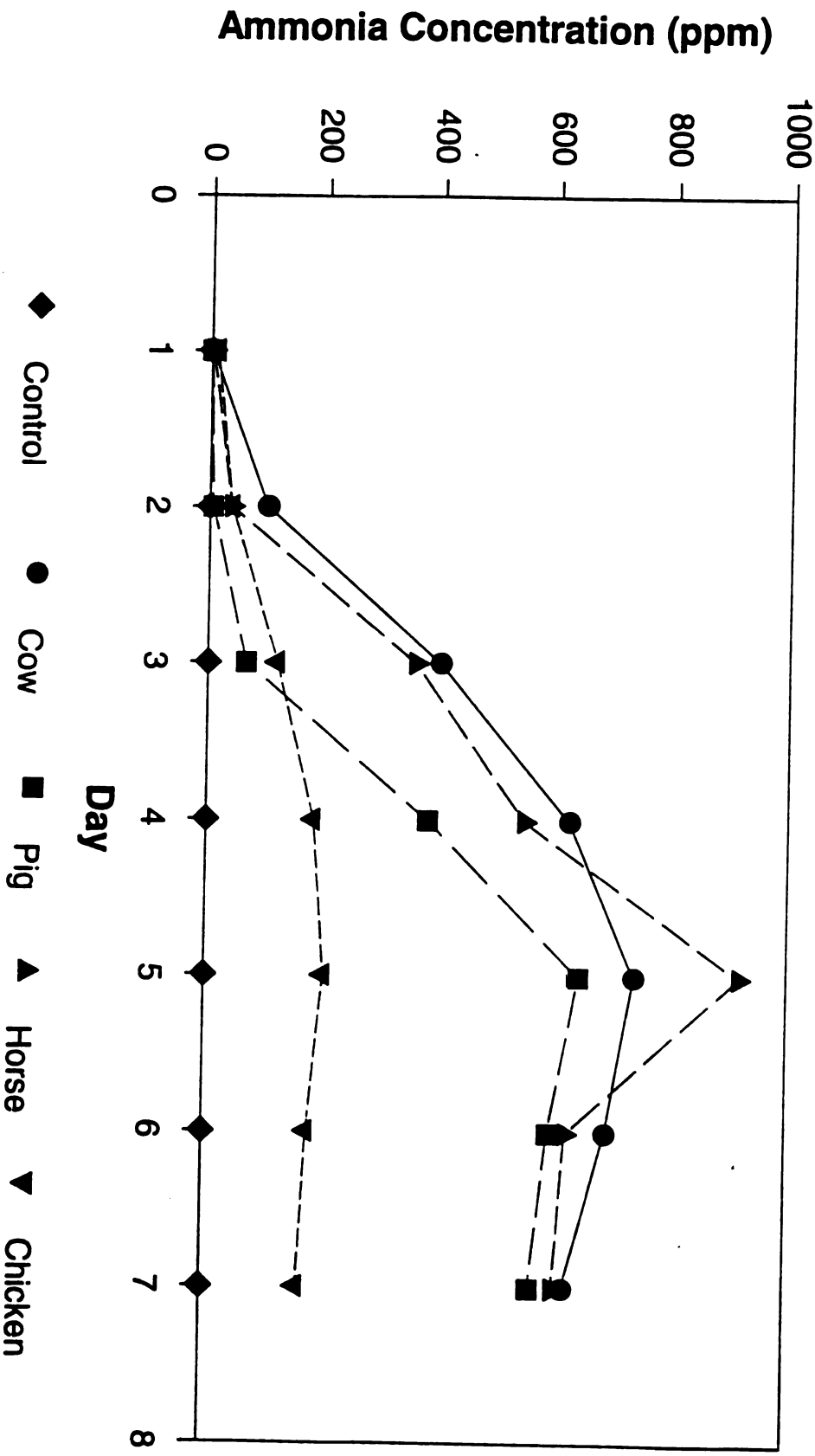
AMMONIA PRODUCTION FROM BEDDING MATERIAL CONTAMINATED WITH DIFFERENT LIVESTOCK URINE

The offensive odor of ammonia is an important environmental problem, particularly in residential areas. Ammonia is produced by the anaerobic decomposition of proteins and a common odor around livestock buildings and waste storages (Tyler, 1978). Contaminated beddings are also considered another major source of ammonia odor production (Barth et al., 1984).

Ammonia production from animal husbandry are part of the N-cycle in agriculture, with 37% of all N losses being in the form of ammonia. Animal production has a nitrogen efficiency of only 17%; and ammonia production from buildings comprise about one-third of the total production of ammonia from animal husbandry (Hartung, 1991).

Ammonia production from sawdust contaminated with cow, horse, pig urine or chicken manure was measured to determine the effect of urine source on ammonia production (Fig.1). Urine source affected ammonia production when the same bedding was used. Ammonia production from the jars containing sawdust contaminated with cow urine increased on the second day (100 ppm), peaked at a maximum on the fourth day (750 ppm) and then slightly decreased as incubation period continued. Ammonia production from sawdust contaminated with pig urine was detected on the third day (67 ppm). Then a rapid and significant increase was observed. The highest production was detected on the fifth day (647 ppm). Finally, when sawdust was contaminated with horse urine, ammonia production increased on the second day (37 ppm)

Figure 1. Ammonia concentration (ppm) produced from sawdust contaminated with either cow, pig, or horse urine and chicken manure. Each point in the curve represents the average of three determinations.


Ammonia Concentration (ppm)
0
200
400
600
800
1000
0
1
2
3
4
5
6
7
8
Day
Control
Cow
Pig
Horse
Chicken

and the highest concentration (920 ppm) was attained on the fifth day (Fig.1). Ammonia production from sawdust contaminated with chicken manure started to increase on the second day (100 ppm), then gradually increased in ammonia production until the fifth day, when the highest ammonia production was reached (200 ppm). There was no significant difference in ammonia production between day 1 and day 2 among species ($P > 0.5$). However there was a significant difference among all other days compared to day 1, the difference was at its maximum at day 5 ($P < 0.001$). It has been reported that urea in animal waste can be enzymatically converted to ammonia in 2 to 5 days; the conversion rates are governed by environmental factors affecting microbial activity (Salter and Schollenberger, 1939). Finally, there was a significant difference ($P < 0.001$) among species, with cow and horse urine being the highest in ammonia production followed by the pig and chicken respectively.

Since sawdust was used as a standard bedding in this study, deviations in ammonia production could be the result of urine content differences, such as urea concentration, ureolytic bacterial numbers and pH. Ureolytic bacterial numbers would affect ammonia production especially in the early days of bedding contamination. Low ureolytic bacterial number in urine would result in low ammonia production. Thus ammonia production would increase only after the bacterial numbers have sufficient time to increase. It was demonstrated in (Table 7, p.122) of this study that the bacterial number in sawdust contaminated with horse urine was high (1.1×10^3). On the other hand,

sawdust contaminated with pig or cow urine contained less ureolytic bacteria ($2.4x10^2$), this might explain the lower ammonia production from cow and pig urine (Fig.1).

Ammonia production from sawdust contaminated with pig urine was low at day two (5 ppm) compared to ammonia production from sawdust contaminated with cow urine at the same day (100 ppm). This may be due to the lower pH (5.4) of pig urine, compared to the pH of cow urine (8.4). It has been reported that low pH results in slower growth of ureolytic bacteria, as well as, lower ammonia production (Seltzer et al., 1969). As the incubation period continued, ammonia production resulted in increasing the pH, allowing better conditions for ammonia production (Seltzer et al., 1969).

Ammonia production from sawdust contaminated with chicken manure showed the lowest reading, whereas, its bacterial content was the highest (Table 7, p. 122), this could be due to the fact that chicken manure contains feces and urine. It has been reported that ammonia production declines when urine is contaminated with manure (Hartung, 1991). Nitrogen in chicken manure is less than nitrogen in the original feed, which is not too unusual, considering that highly utilizable protein in rations for laying hens are transferred as egg protein and that some volatilization of ammonia-nitrogen in the manure may occur quite rapidly after excretion (Azevedo and Stout, 1974).

Mean ammonia production from sawdust contaminated with cow, horse, and pig urine or chicken manure was 455, 443, 322 and 129 ppm respectively.

It has been reported that a great proportion of water eliminated by horses and cattle is excreted in the feces; whereas pigs excrete most waste water via urine. Proportionally less dry matter is excreted in the urine of pigs than other animals, so pig urine appears to be especially diluted. The feces and urine of poultry are excreted as one mass. Dixon (1958) determined that about 55 to 58% of fresh poultry manure was urine, which contained about 0.40% nitrogen. This difference in H_2O content of urine might explain the variation in ammonia production among species.

This data suggests that increasing the ventilation, changing and/or treating the contaminated bedding at day 5 (where ammonia production reached its maximum) seems to be a good idea to decrease ammonia production from livestock barns. However, as ventilation rate increases, a greater amount of odorous, volatile compounds are likely to be released from the waste as it gets dried (Dorling, 1977). The increased number of odorous molecules could be less concentrated as a result of the greater air flow, and hence could create less of a nuisance. In addition, the difference in urine characteristics (water, urea, bacterial numbers and species content) in many animals would result in different levels of ammonia production.

THE INHIBITORY EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON AMMONIA PRODUCTION FROM DIFFERENT BEDDING MATERIALS CONTAMINATED WITH LIVESTOCK URINE

Ammonia Production From Beddings Contaminated With Cow Urine.

Ammonia production from bedding materials contaminated with cow urine (Fig. 2-7) indicated that stripped newspaper bedding (Fig. 2) had some variation in the ammonia production of the control (range 17-164 ppm). The addition of pine oil seemed to exacerbate ammonia production in the same bedding (range 27-177 ppm), this increase however was not significant ($P > 0.5$). Boric acid showed a lower ammonia production (range 11-96 ppm), but it was nonsignificant ($P > 0.5$). However pine oil and boric acid combination showed a significant ($P < 0.02$) reduction in ammonia production compared to the control. With corncobs (Fig.3), some white fungal growth was observed on the top of the bedding in the jars. The combination of boric acid and pine oil showed low ammonia level (range 13-97 ppm), even lower than boric acid alone (range 10-133 ppm). The decreased ammonia production with both boric acid and the combination were highly significant ($P < 0.003$)compared to the control. Pine oil ammonia production ranged between (range range 33-177 ppm) and showed some inhibition of ammonia production (P > 0.5), but not substantially over the control (range 33-190 ppm). With sawdust, (Fig. 4), the combination of boric acid and pine oil was more effective in reducing ammonia production (range 52-240 ppm) ($P < 0.05$), than either pine oil (range 130-370 ppm) ($P > 0.5$) or boric acid (range 55-300 ppm) ($P > 0.5$) alone. Again, pine

Figure 2. Ammonia concentration (ppm) produced from newspaper contaminated with cow urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

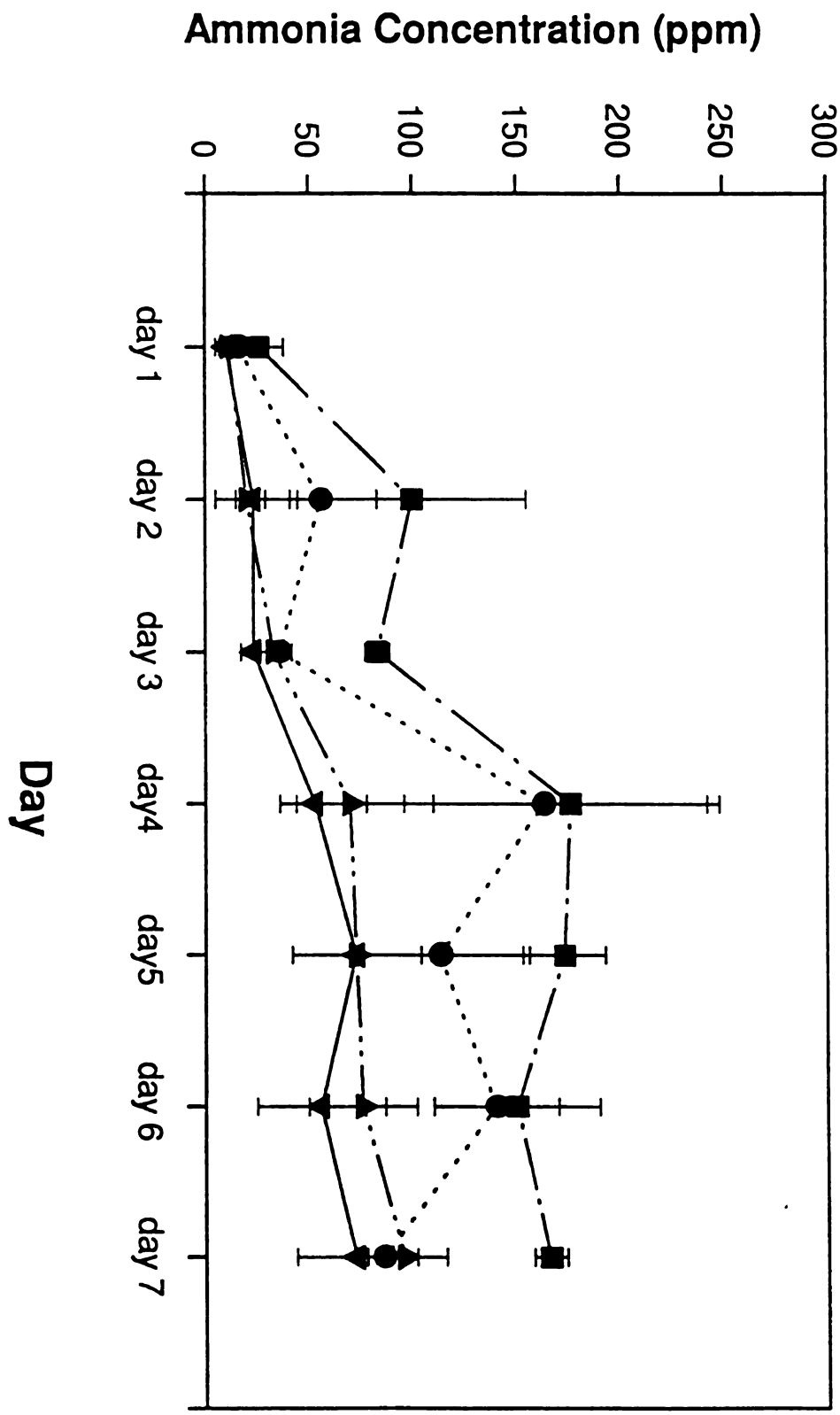

Ammonia Concentration (ppm)
0
50
100
150
200
250
300
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 3. Ammonia concentration (ppm) produced from corncobs contaminated with cow urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

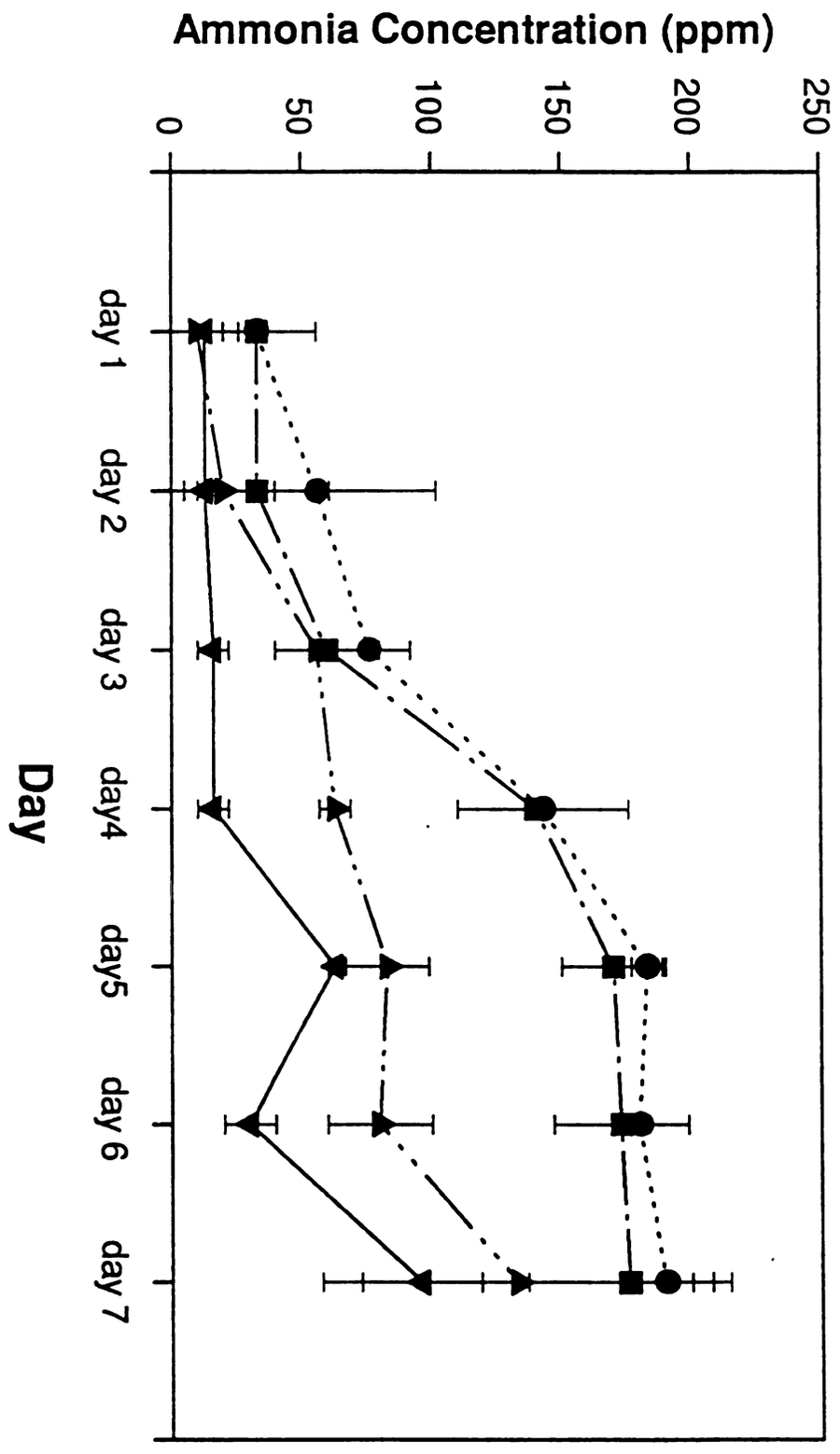

Ammonia Concentration (ppm)
0
50
100
150
200
250
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 4. Ammonia concentration (ppm) produced from sawdust contaminated with cow urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

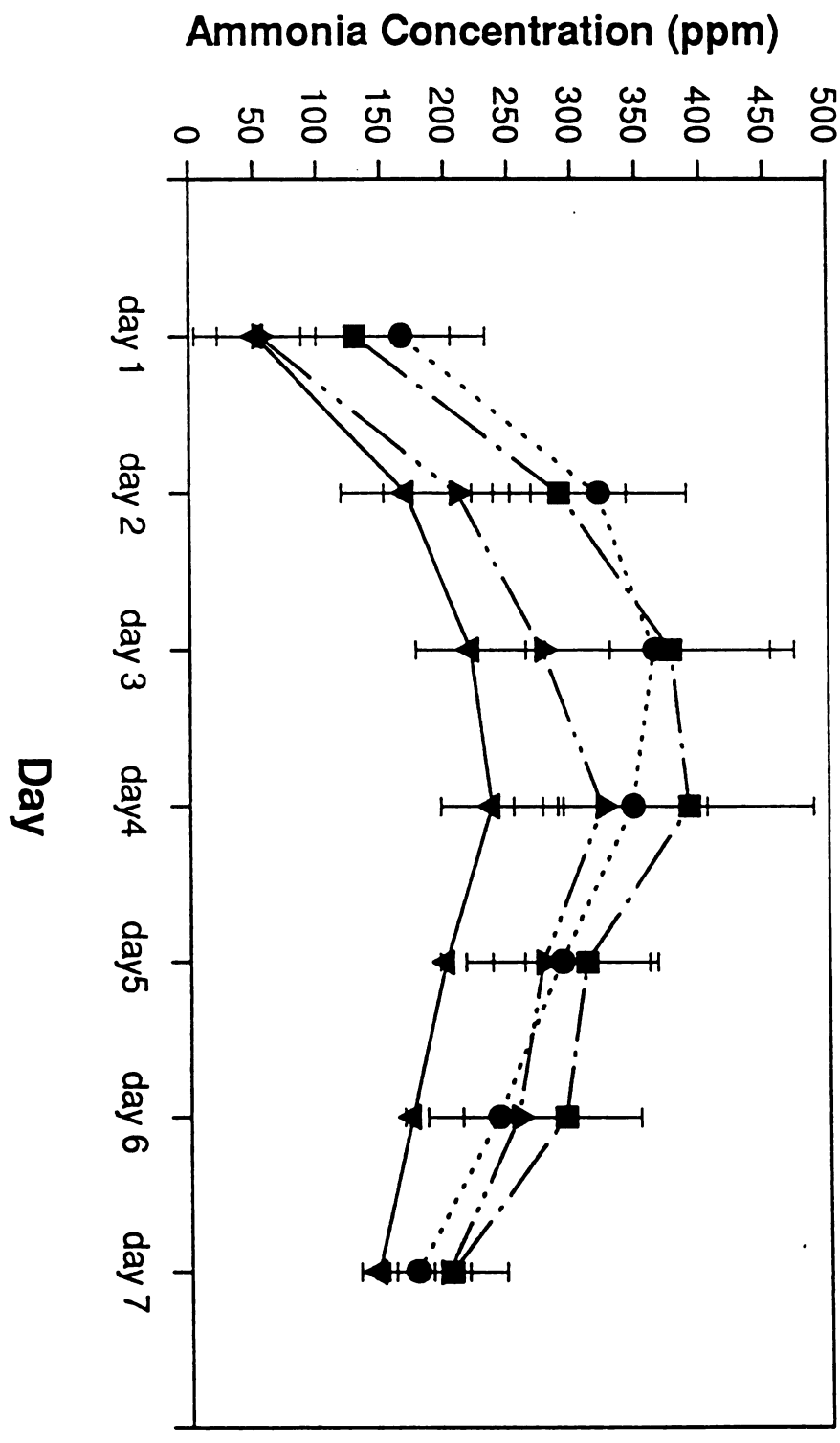

Ammonia Concentration (ppm)
0
50
100
150
200
250
300
350
400
450
500
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 5. Ammonia concentration (ppm) produced from sand contaminated with cow urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

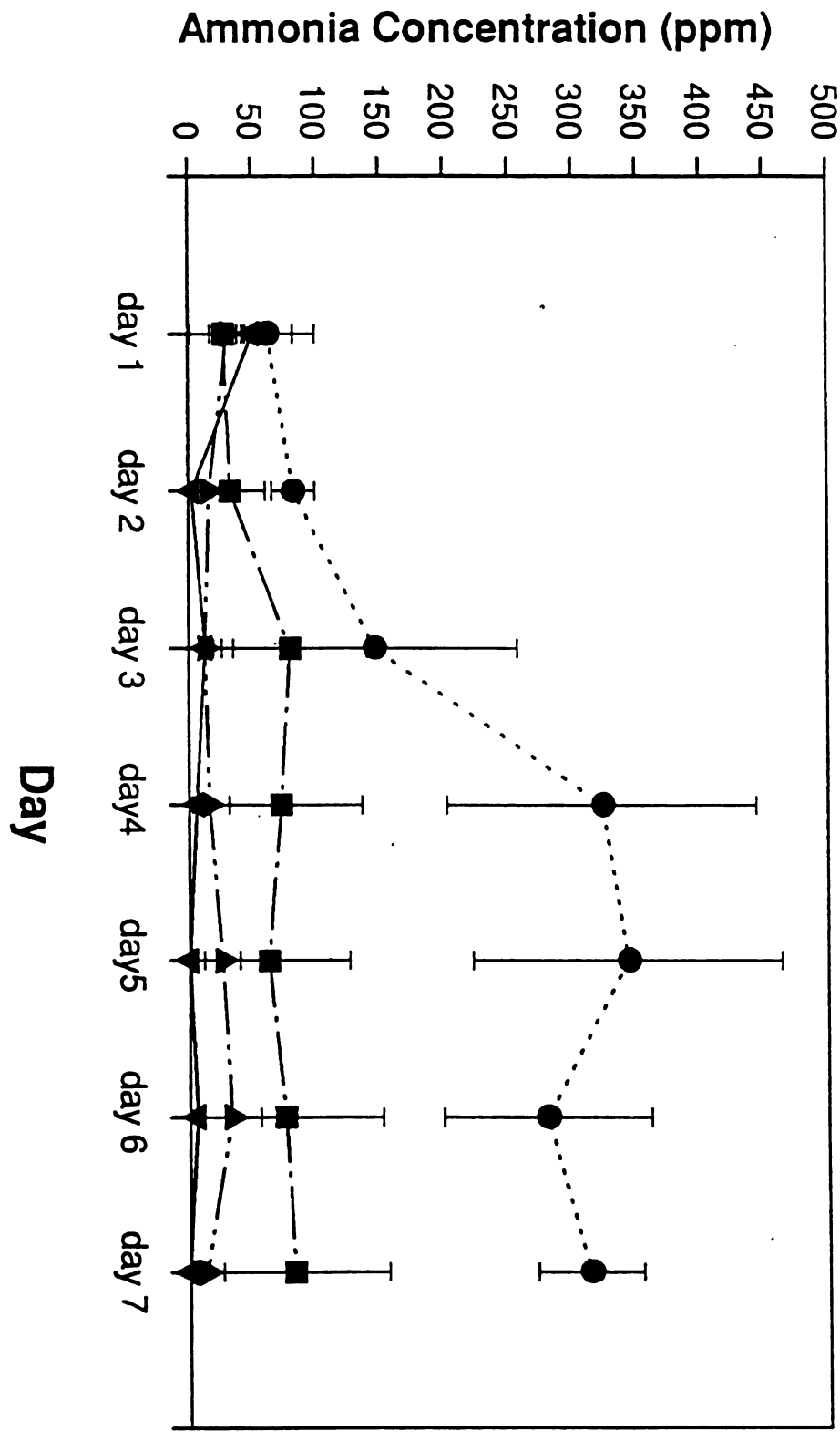

Ammonia Concentration (ppm)
0
50
100
150
200
250
300
350
400
450
500
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 6. Ammonia concentration (ppm) produced from woodshaving contaminated with cow urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

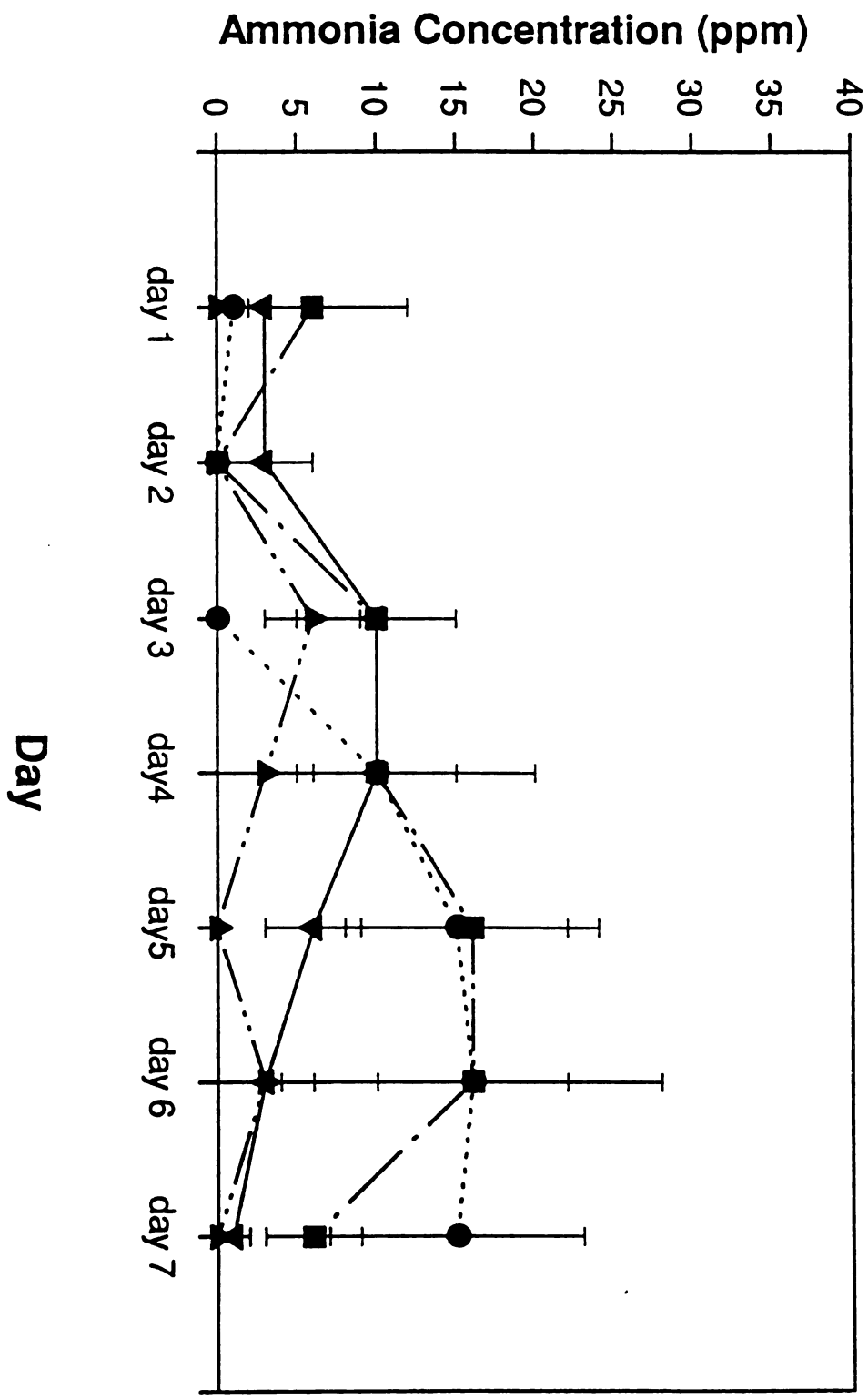
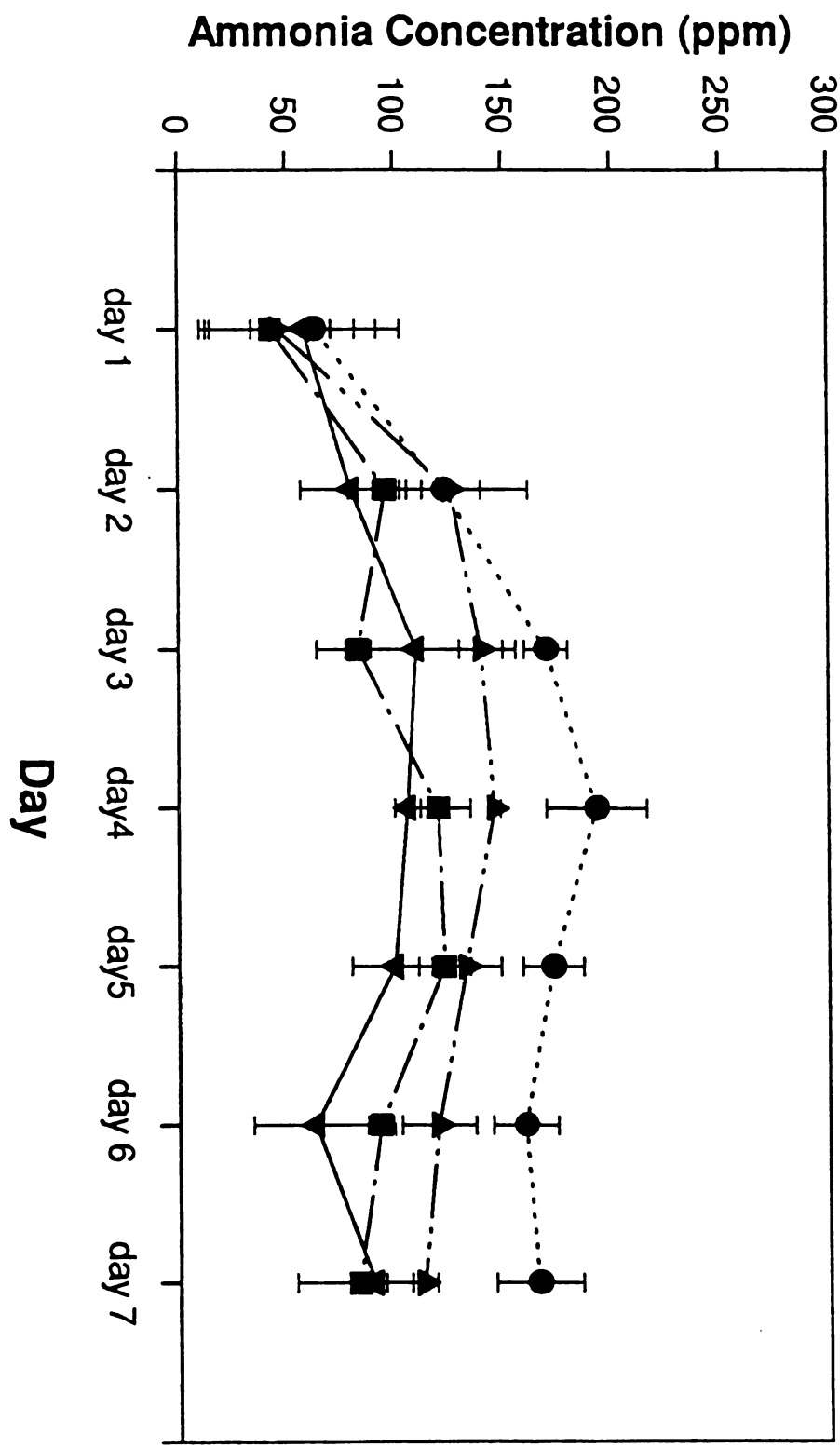

Ammonia Concentration (ppm)
0
5
10
15
20
25
30
35
40
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 7. Ammonia concentration (ppm) produced from straw contaminated with cow urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).


Ammonia Concentration (ppm)
0
50
100
150
200
250
300
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

oil alone seemed to exacerbate ammonia production in sawdust bedding (range 130-370 ppm). With sand (Fig. 5), all treatments were highly effective in reducing ammonia production ($P < 0.001$) compared to the control. The combination (range 0-50 ppm) and boric acid (range 13-33 ppm) were very effective treatments. Also, pine oil (range 28-75 ppm) was also effective over the control (range 63-313 ppm). Woodshaving (Fig. 6), showed a very low and erratic ammonia production when contaminated with cow urine, since ammonia production never attained 20 ppm during the 8 days of incubation. Boric acid (range 0-7 ppm) was more significant ($P < 0.02$) than the combination (range 2-10 ppm) ($P < 0.3$), and both were more effective than pine oil (range 0-17 ppm) ($P > 0.5$). Woodshaving seemed to either contain an unknown inhibitory substance(s) or other unique properties that resulted in decreased ammonia production. With straw (Fig. 7), boric acid and the combination were effective in reducing ammonia production. Boric acid readings were (range 46-147 ppm) ($P < 0.02$) and the combination (range 59-110 ppm) ($P < 0.001$). Pine oil (range 43-123 ppm) seemed to be more effective than boric acid ($P < 0.001$), but the differences were small.

Ammonia Production From Beddings Contaminated With Horse Urine.

Ammonia production curves obtained from beddings contaminated with horse urine (Fig. 8-11) indicated that the combination (range 0-20 ppm) and boric acid (range 0-13 ppm) treatments were very effective in decreasing ammonia production from sand (Fig. 8). Pine oil (range 27-80 ppm) was

Figure 8. Ammonia concentration (ppm) produced from sand contaminated with horse urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

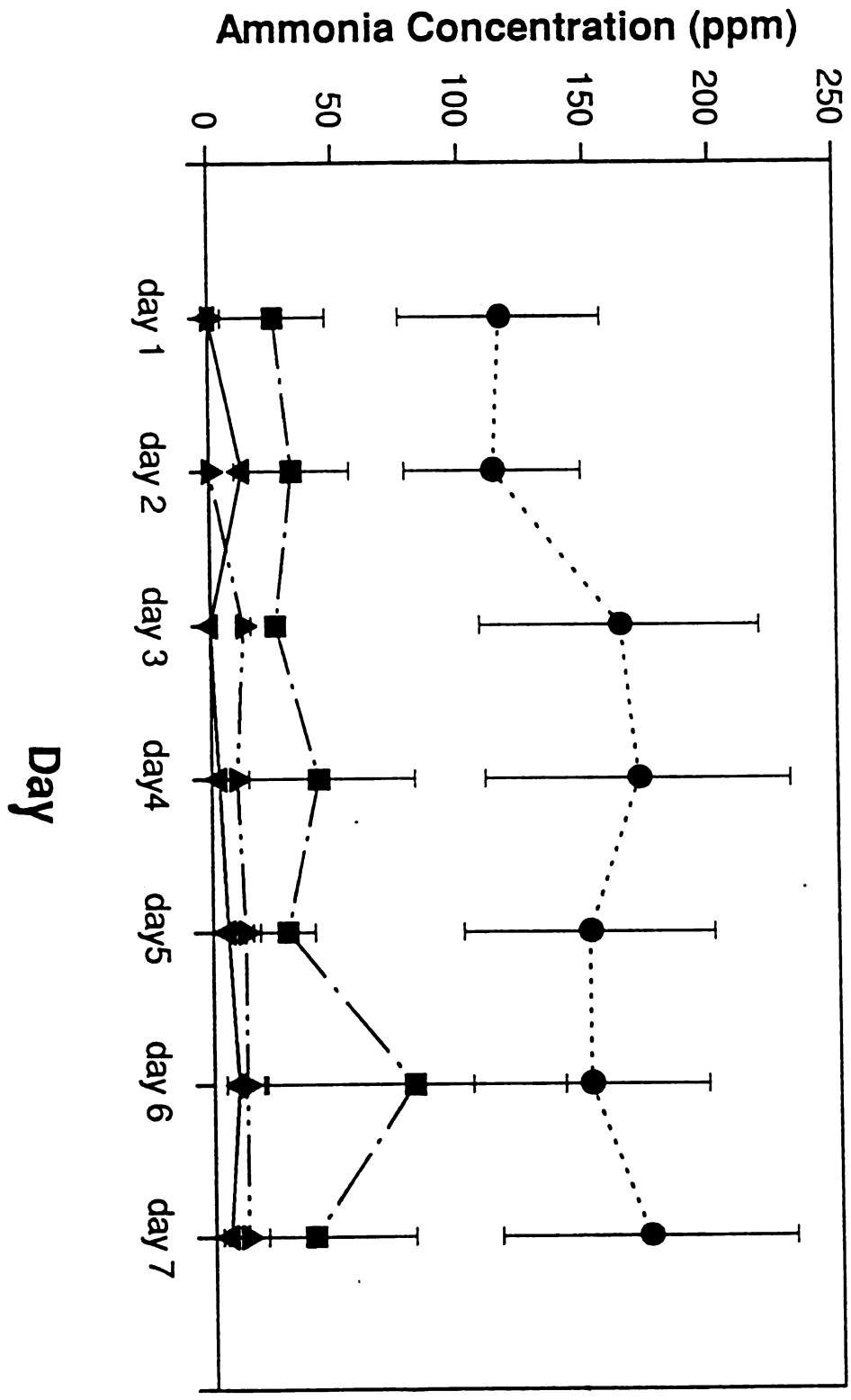

Ammonia Concentration (ppm)
0
50
100
150
200
250
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 9. Ammonia concentration (ppm) produced from sawdust contaminated with horse urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

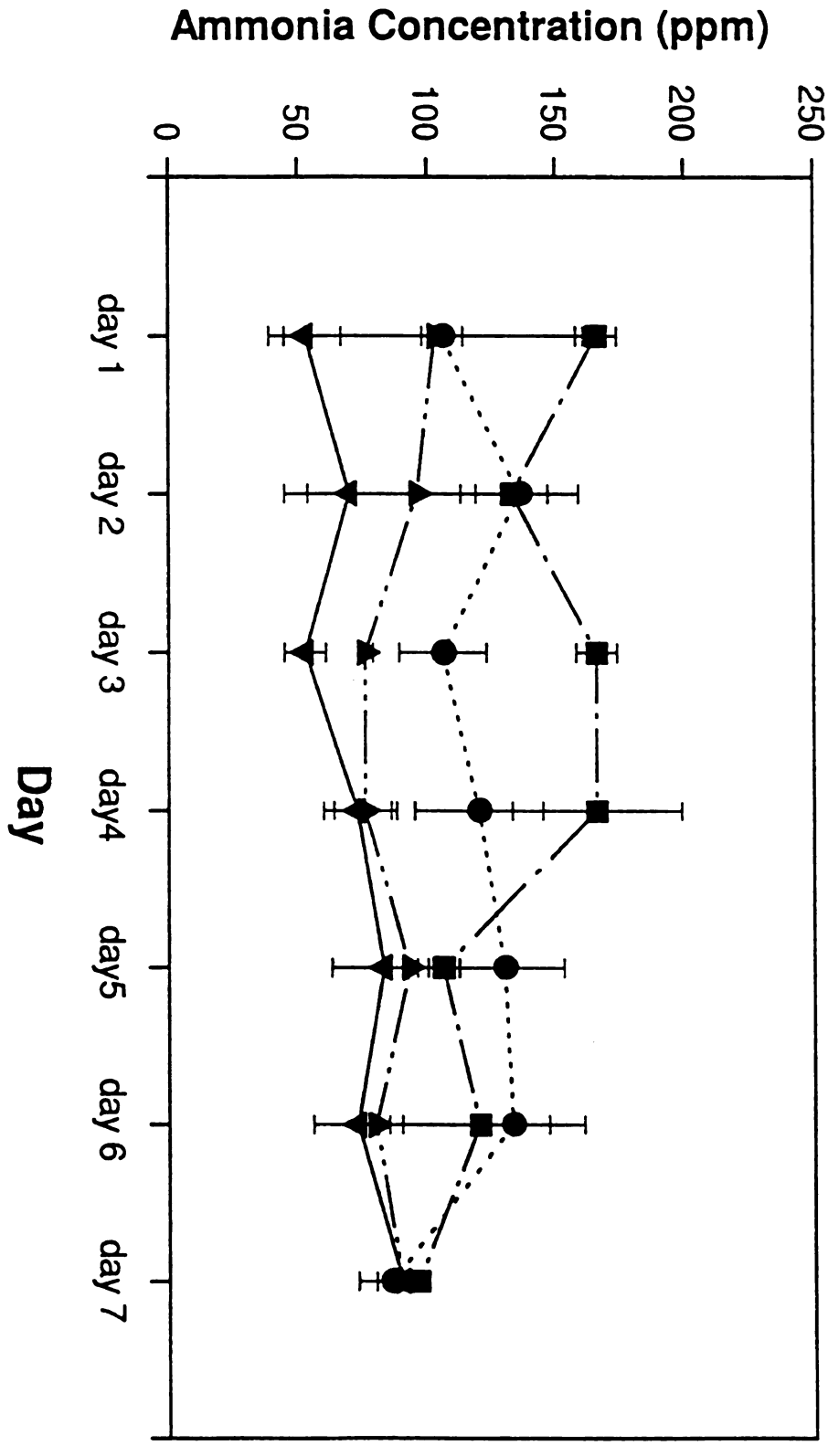

Ammonia Concentration (ppm)
0
50
100
150
200
250
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 10. Ammonia concentration (ppm) produced from straw contaminated with horse urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

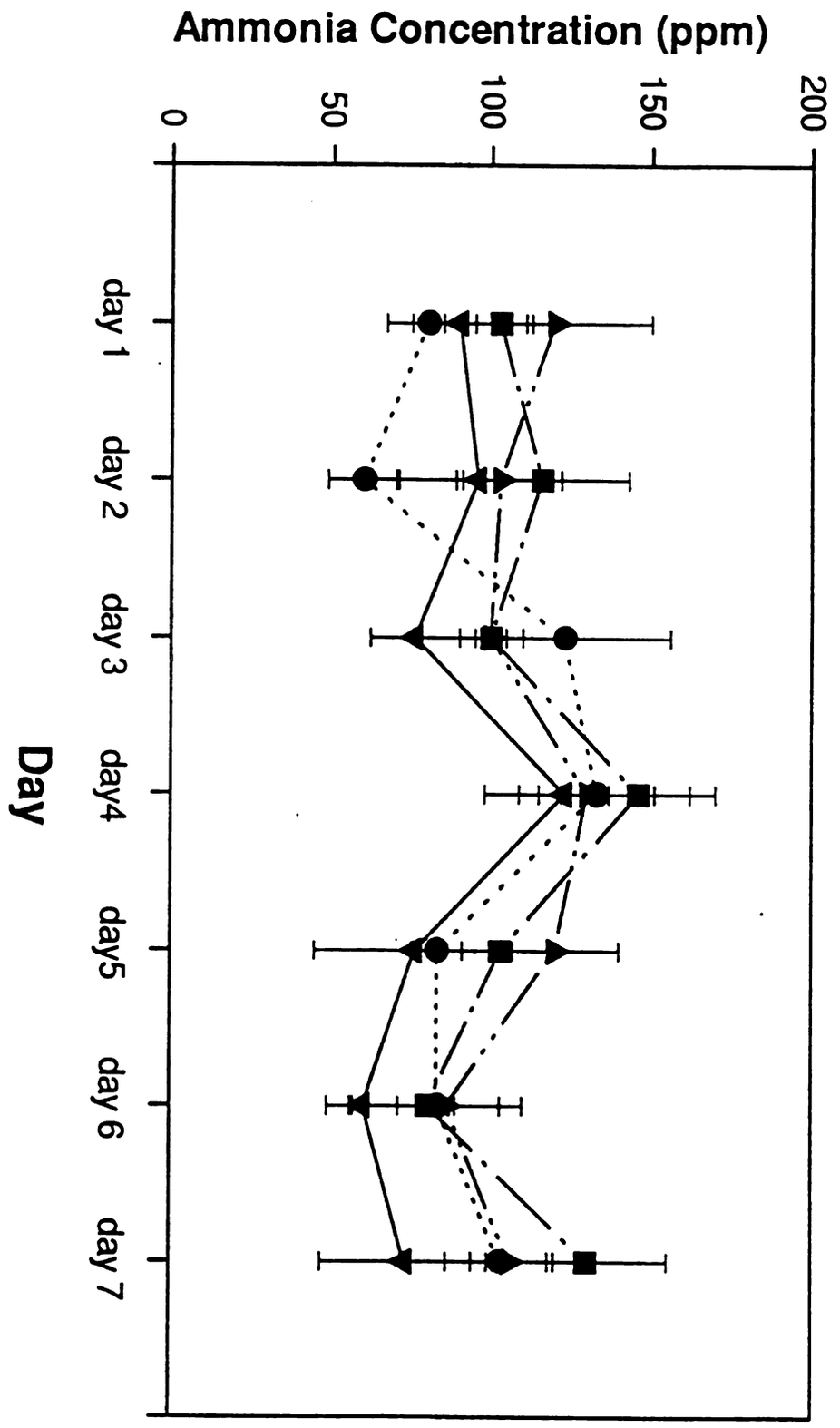
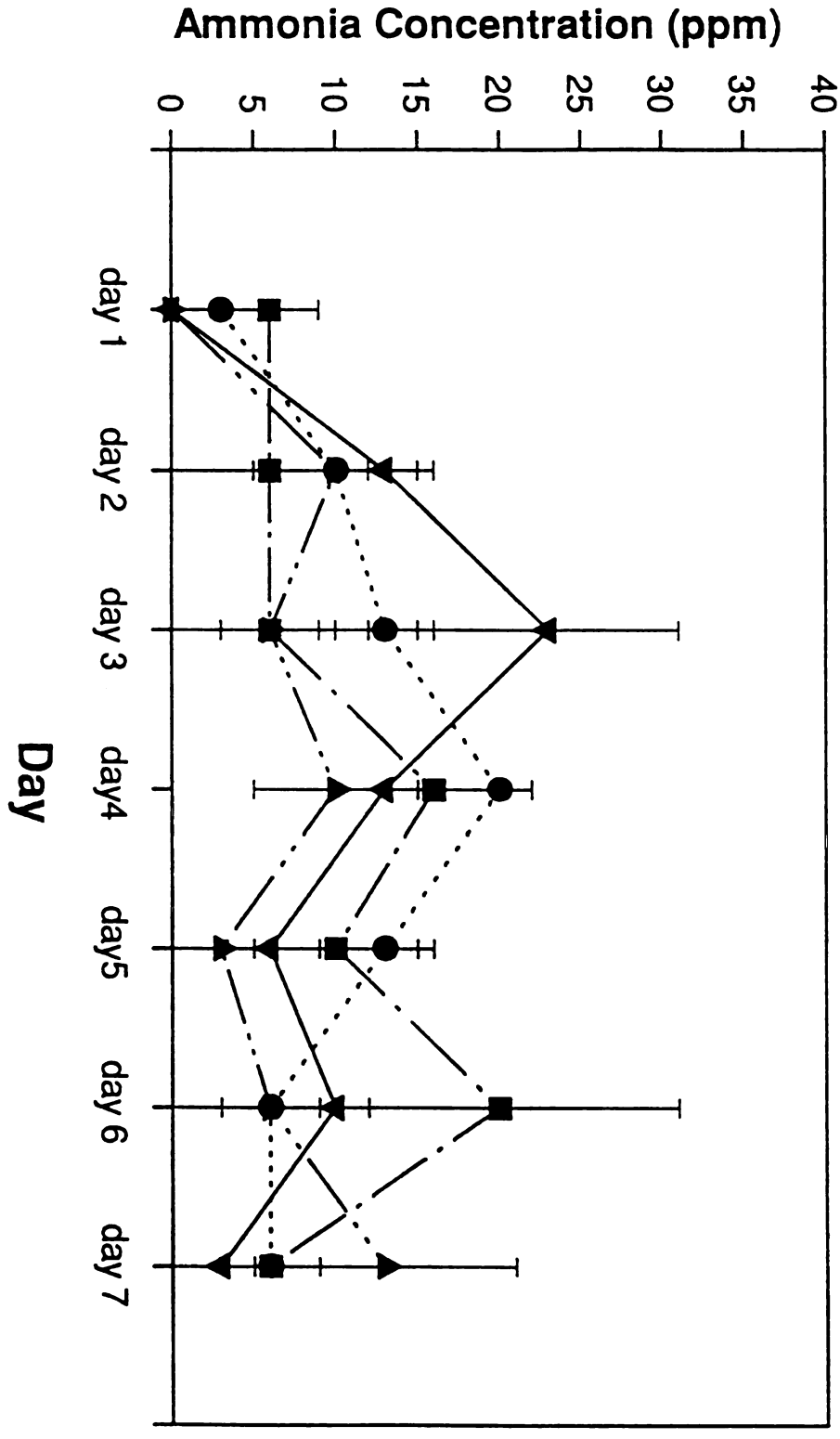

Ammonia Concentration (ppm)
0
50
100
150
200
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 11. Ammonia concentration (ppm) produced from woodshaving contaminated with horse urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

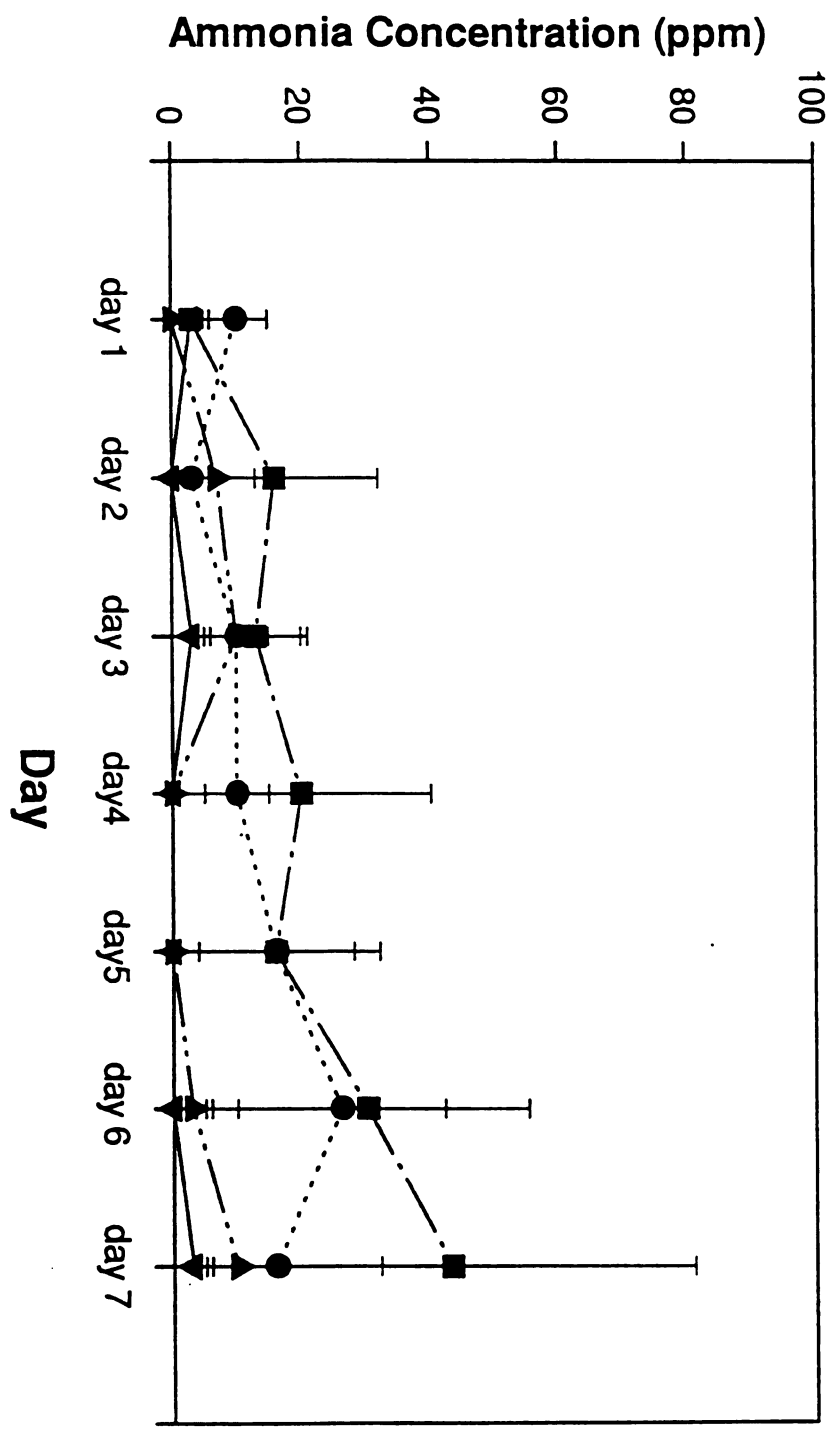

Ammonia Concentration (ppm)
0
5
10
15
20
25
30
35
40
Day
day 1
day 2
day 3
day4
day5
day 6
day 7

effective in comparison to the control (range 117-173 ppm), but not as effective as either the combination or boric acid. All treatments were highly significant ($P < 0.0001$) in reducing ammonia production. With sawdust (Fig. 9) the combination (range 53-90 ppm) and boric acid (range77-103 ppm) were the most effective ($P < 0.001$ and $P < 0.012$ respectively), but not as effective as in sand. Pine oil (range 97-166 ppm) treatment did not seem to be effective ($P > 0.5$) in sawdust, and may even exacerbate ammonia production. In straw (Fig.10) ammonia curves are biphasic for all treatments ($P > 0.5$). Boric acid (range 86-130 ppm) did not seem to have strong inhibitory effect. Pine oil (range 80-146 ppm) was not effective, whereas, the combination (range 60-123 ppm) showed some effect. With woodshaving (Fig.11), ammonia production was very erratic with all concentrations detected below 25 ppm over 8 consecutive days. This depressed response could be attributed to the inhibitory substance(s) in woodshaving. Boric acid (range 0-13 ppm) seemed to be effective ($P < 0.25$), but the combination (range 0-23 ppm) and pine oil (range 7-20 ppm) were not effective ($P > 0.5$). Since all treatments were significantly depressed, a comparative evaluation was difficult to make.

Ammonia Production From Beddings Contaminated With Pig Urine.

When sand was contaminated with pig urine (Fig.12) the combination (range 0-3 ppm) and boric acid (range 0-10 ppm) treatments, were very effective ($P < 0.04$ and $P < 0.09$ respectively) in inhibiting ammonia production. Pine oil treatment alone appeared to be less effective ($P < 0.23$)

Figure 12. Ammonia concentration (ppm) produced from sand contaminated with pig urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).


Ammonia Concentration (ppm)
0
20
40
60
80
100
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

in comparison to the control. All ammonia concentrations were below 50 ppm. The results obtained with woodshaving (Fig.13), showed that all ammonia concentrations were low (below 12 ppm). The combination and boric acid treatments were effective ($P < 0.05$), while pine oil was not ($P > 0.5$). Also, in sawdust (Fig.14) boric acid and the combination, were effective ($P < 0.005$) in inhibiting ammonia production, but the pine oil treatment alone was less effective ($P < 0.24$).

Ammonia Production From Beddings Contaminated With Poultry Manure.

Ammonia production from poultry manure mixed with woodshaving (Fig.15) indicated that boric acid (range 7-27 ppm) and the combination (range 13-40 ppm) were more effective ($P < .001$ and $P < 0.03$ respectively), than pine oil (range 20-43 ppm) alone ($P > 0.5$). With sawdust (Fig.16), boric acid (range 57-83 ppm) and the combination (range 38-80 ppm) were the most effective ($P < 0.001$) in inhibiting ammonia production. However, pine oil (range 80-160 ppm) alone appeared to be ineffective ($P < 0.5$).

Beddings And Species Effect On Ammonia Production.

Since sawdust and woodshaving were used in all species, the data of these two beddings was compared within each species to know which bedding is more effective in reducing ammonia production. Ammonia production from woodshaving contaminated with urine was significantly ($P < 0.001$) lower than sawdust. The data was also used to study effect of urine source, where ammonia production from each species within the same bedding (sawdust) was

Figure 13. Ammonia concentration (ppm) produced from woodshaving contaminated with pig urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

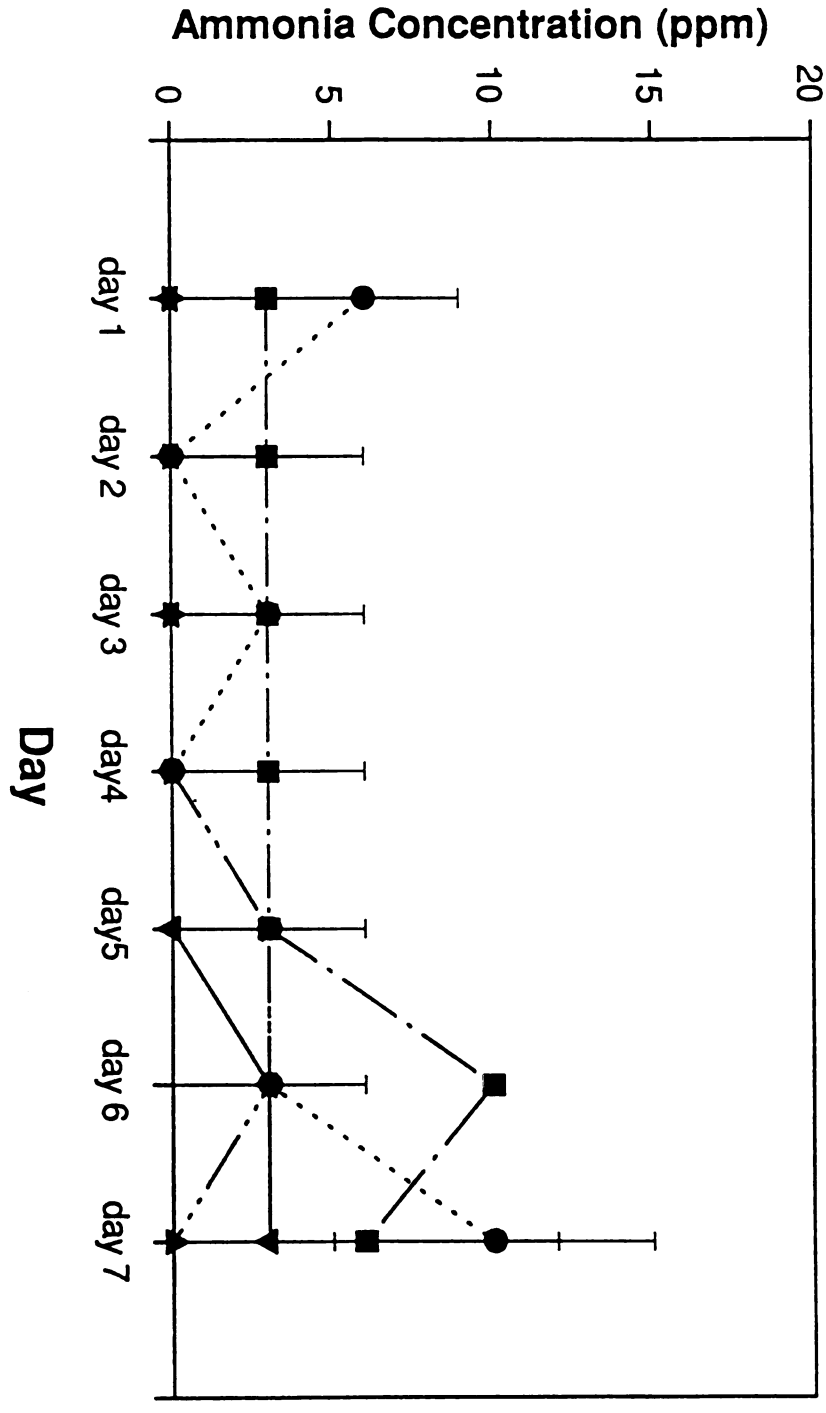

Ammonia Concentration (ppm)
0
5
10
15
20
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 14. Ammonia concentration (ppm) produced from sawdust contaminated with pig urine. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

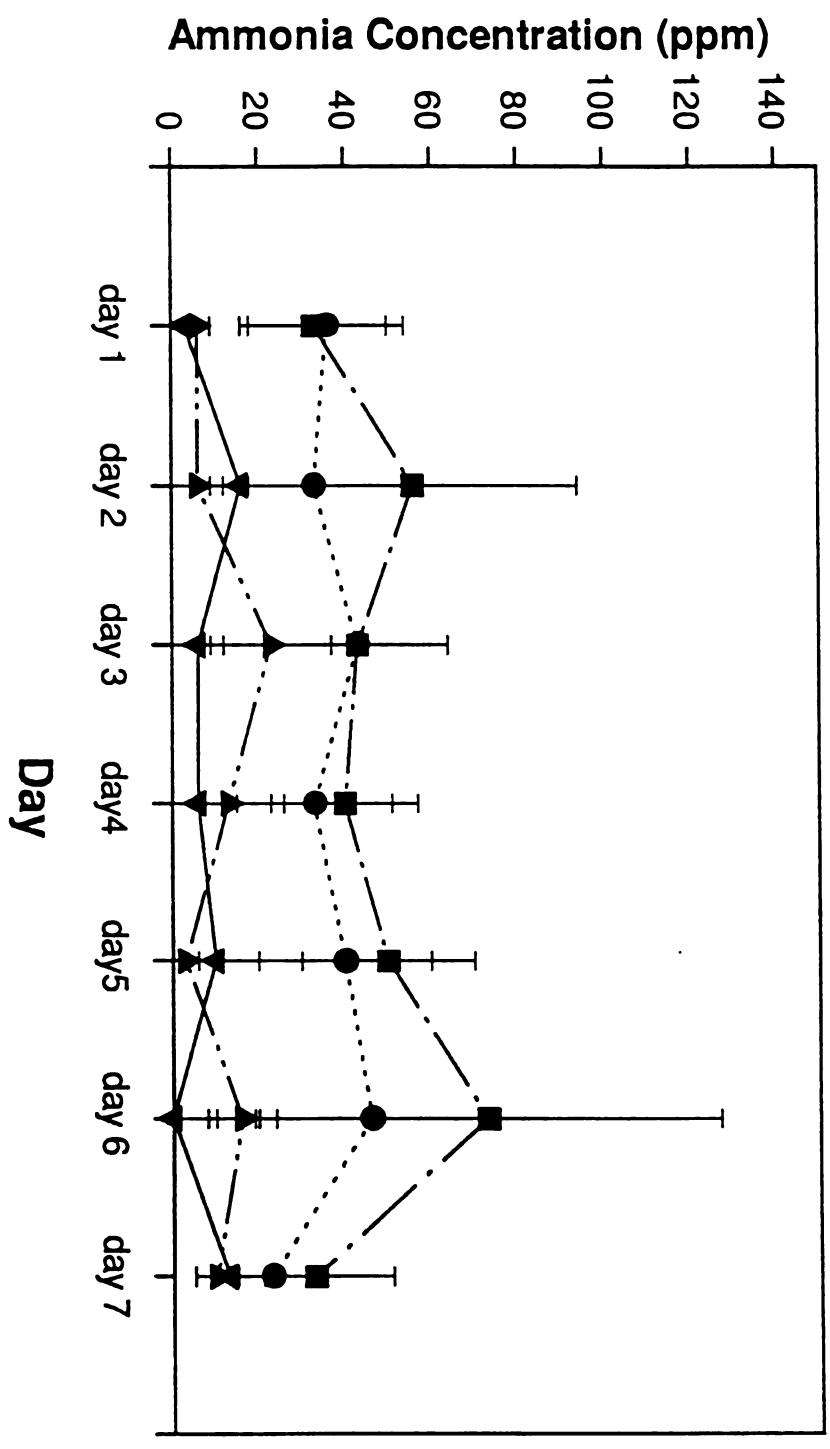

Ammonia Concentration (ppm)
0
20
40
60
80
100
120
140
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 15. Ammonia concentration (ppm) produced from woodshaving contaminated with chicken manure. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).

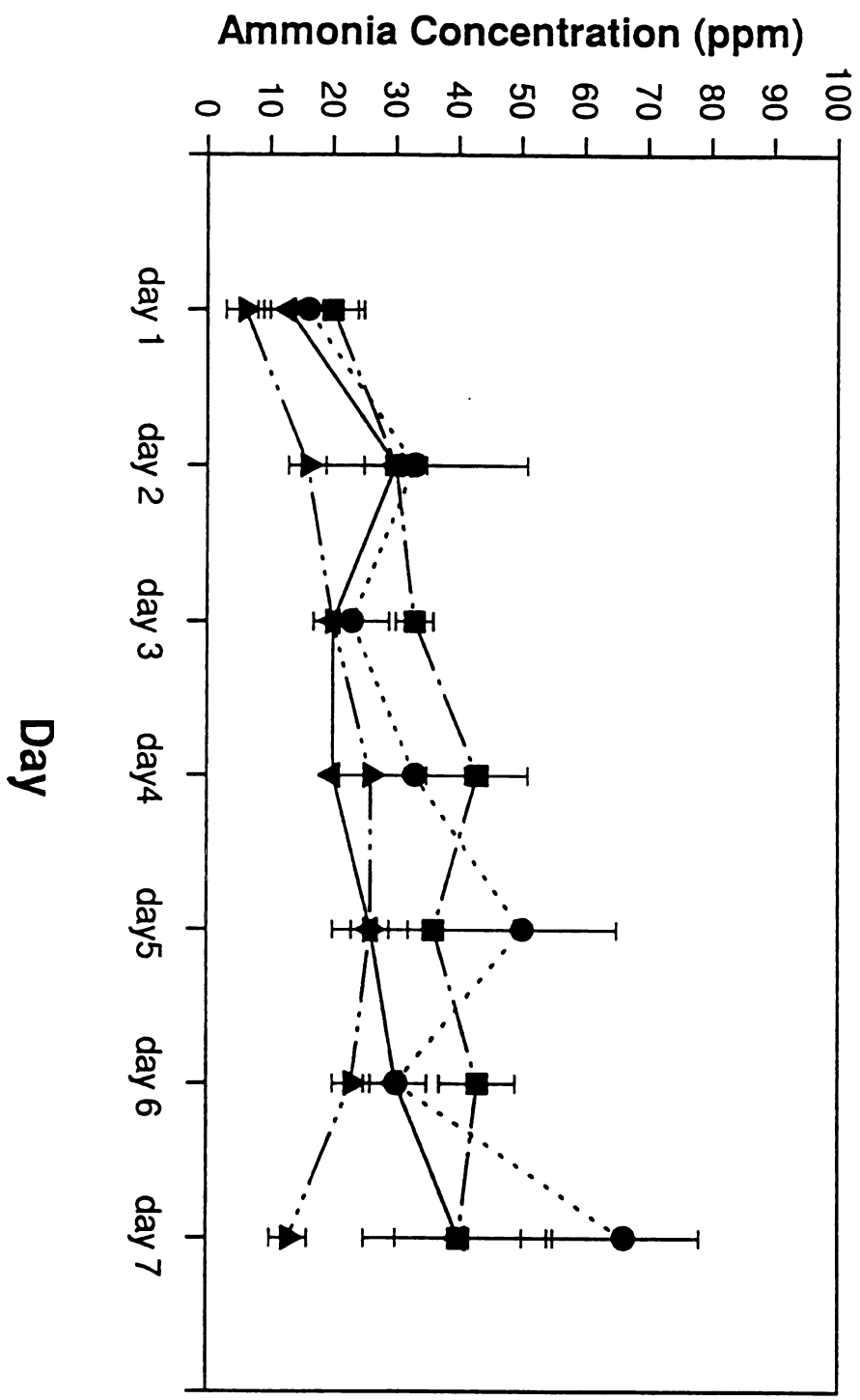
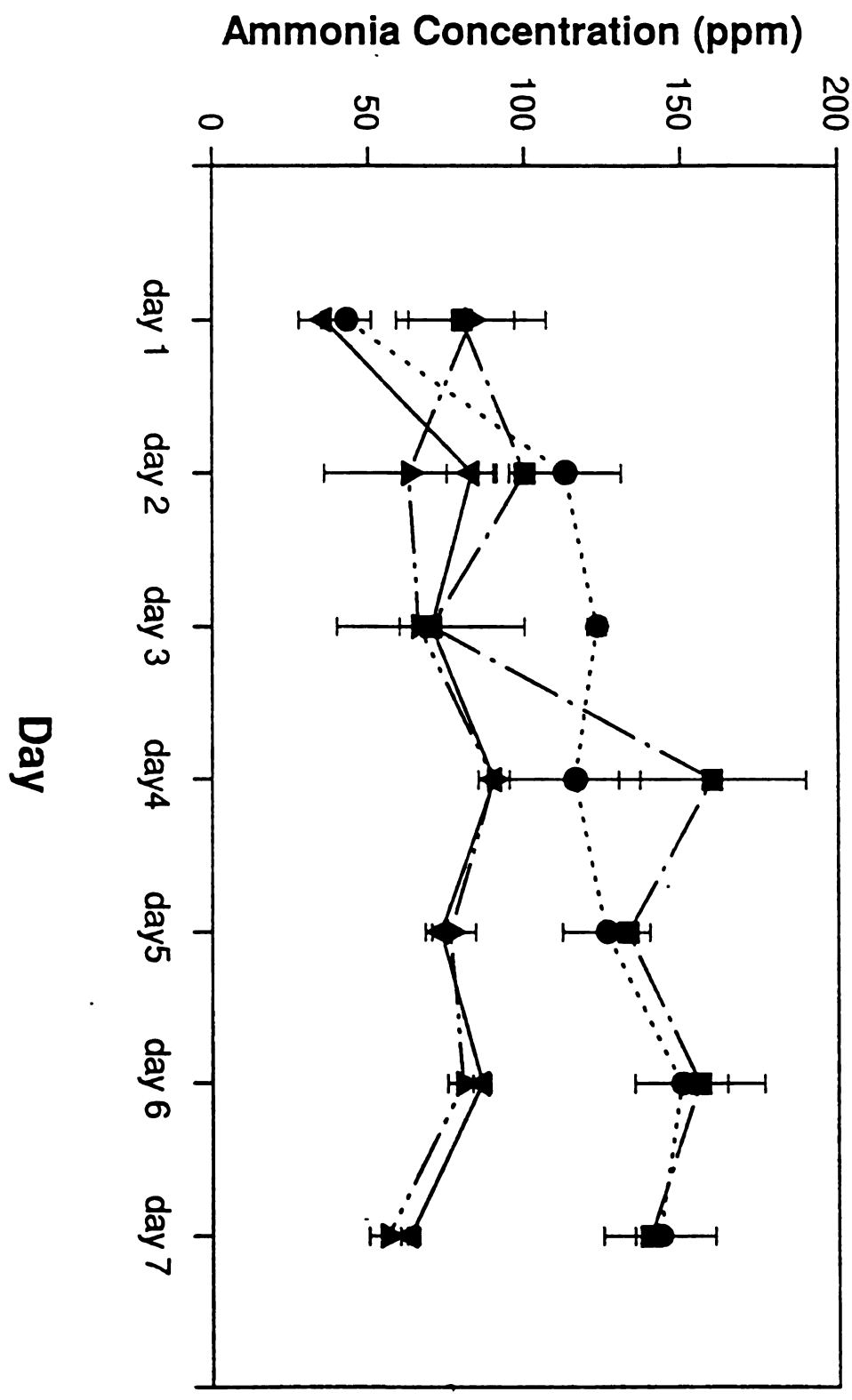

Ammonia Concentration (ppm)
0
10
20
30
40
50
60
70
80
90
100
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

Figure 16. Ammonia concentration (ppm) produced from sawdust contaminated with chicken manure. Contaminated bedding was treated with pine oil (PO), boric acid (B) and their combination (M). Control received carrier solution. Each point in the curve represents the average of three determinations. (●) Control, (■) Pine oil, (▲) Boric Acid, (▼) Combination (Pine oil + Boric acid).


Ammonia Concentration (ppm)
0
50
100
150
200
day 1
day 2
day 3
day4
day5
day 6
day 7
Day

compared. Statistical analysis showed a significant effect ($P < 0.001$) for urine source on ammonia production. When woodshaving was used ammonia production was the highest in poultry, followed by horse, cow and pig in a descending order. However, when sawdust was used, ammonia production was the highest in cow, followed by horse, pig and chicken in a descending order.

Differences in urine contents among species could explain the effect of urine source on ammonia production. A large proportion of water eliminated by horse and cattle is excreted in feces, so horse and cattle urine is more concentrated, whereas swine excrete most waste water via their urine. Proportionally less dry matter is excreted in the urine of swine than other animals, so swine urine appears to be especially diluted (Salter and Schollenberger, 1939). The excretion of urine by hogs of 51 to 90 Kg body weight ranged from 2.9 to 4.0 liters/head/day respectively. Pig urine excreted contained 0.4% nitrogen. Poultry manure has a basic pH (7.68), 80-90% moisture and 82% of the nitrogen was organic. Inorganic nitrogen was mostly ammoniacal (Nodar et al., 1990). These differences in urine contents would obviously affect urea concentration in urine as well as the type and number of ureolytic bacteria; which results in different ammonia production rates.

Bedding effects on ammonia production was tested by comparing ammonia production from the diferent beggings used in the control of each species. Different beddings showed significant effect ($P < 0.001$) in

decreasing ammonia production in all species. Ammonia production from control treatment within each species showed different effects for different beddings. With cow urine, the order of increasing inhibition of ammonia production was sawdust, sand, straw, corncobs, newspaper and woodshaving. With pig urine, the order of increasing inhibition of ammonia production was sawdust, sand and woodshaving. With horse urine, the order of increasing inhibition of ammonia production was sand, sawdust, straw and woodshaving, while with chicken manure it was sawdust then woodshaving.

The two properties of bedding likely to exert the greatest influence on odor production are its absorption and physical structure. Most bedding materials such as chopped straw, woodshavings and shredded newspaper are highly absorbent and therefore tend to dry the waste (O'Neill et al., 1991). Straw or other bedding material retain urine and prevent the loss of ammonia. As a result, ammonia production from these bedding materials would be low when they are contaminated with livestock urine. In the case of chicken manure, two-thirds of poultry manure nitrogen can be lost as ammonia if litter is not used, but only one-third may be lost if droppings fall on litter (Eno,1966). Therefore, the use of bedding materials would reduce ammonia production from chicken manure. Farmyard manure is produced when chopped straw is used as a bedding. It has been reported that farmyard manure has a more acceptable smell than the waste by itself (Williams et al., 1981). This may be due to the physical structure of straw encouraging aerobic decomposition, thereby

inhibiting anaerobic decomposition and thus the production of more malodorous compounds.

The results obtained with woodshaving suggest that the woodshavings used contained an unknown inhibitory substance(s). The substances were not identified, but they may be phenolic compounds. Phenolic compounds exert maximum vapor pressure at normal pH of fresh slurry (pH 7) which contributes to its characteristic odor (Phillips et al, 1979).

Any means of preventing the development of anaerobic conditions in the waste is likely to reduce the odor problem. In particular, prompt removal of the waste from buildings altogether is a certain way of avoiding production of very offensive odors associated with anaerobic decomposition. Other measures to reduce ammonia production from buildings like the use of bedding materials (Kowalewsky, 1981) depend very much on the amount and maintenance of the bedding.

The use of different bedding materials, as a means of abating odor does not yet seem to have been investigated scientifically; the evidence to date is only anecdotal. The need for such information is increasing, both because the welfare recommendations for livestock (Anon, 1983) encourage the use of bedding, and because other abatement techniques are costly.

In summary, boric acid and combination (boric acid and pine oil) were the most effective treatments in inhibiting ammonia production, regardless of livestock species and bedding material. Boric acid and the combination seemed

to be most effective in sand. There seemed to be some evidence for a boric acid and pine oil interaction (i.e. cow urine/corncobs and sawdust). However, pine oil did not seem to be particularly effective with several bedding materials (e.g. straw, woodshaving, sawdust, and newspaper). The combination interaction might be explained by an indirect effect of pine oil either serving as a surface active agent to facilitate the uptake of boric acid, or somehow reacting with boric acid to make it more effective. The inhibitory effects of pine oil did not appear to be significant ($P > 0.5$), except when sand was used ($P < 0.5$).

Generally, the most certain way of avoiding odor nuisance from a livestock building is the frequent removal of waste, as well as good natural ventilation and good choice of bedding material appropriate to each species. The results of this study justify a continuation of the treatments (boric acid, pine oil and their combination) testing is an effective way to reduce ammonia production.

THE INHIBITORY EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON THE GROWTH OF PURE CULTURES OF UREOLYTIC BACTERIA ISOLATED FROM CONTAMINATED LIVESTOCK BEDDINGS.

The diffusion method (Petersdorf and Sherris, 1965) was used to study the effect of the treatments (boric acid, pine oil and their combination) on ureolytic bacteria growth. Diffusion susceptibility test depends on the ability of a treatment to be released from a reservoir, such as a cut well in an agar medium, thus creating a circular diffusion gradient of the treatment radiating around the well. The diffusion method may be affected by a variety of factors including the nature of the treatment, the density of the agar relative to the speed of diffusion and with some treatments, the ionic concentration of the medium. The distance of the inhibitory zone around the microorganism being tested is also partially determined by the amount of microbial growth that has occurred by the time an inhibitory concentration of the treatment has diffused into the agar (Cooper and Gillespie; 1952).

The size of the inoculum has actually been found to be the most important variable influencing the distance of the inhibition zone because the position of its border is determined when the critical cell mass is attained (Barry, 1980). Obviously, the more microorganisms provided in the inoculum, the greater the opportunity for generating visible growth before the antimicrobial agents reach inhibitory concentrations at a given distance from the well. Conversely, the fewer microorganisms used to seed the plate, the lesser their chances for producing visible growth at a particular distance before inhibition

takes over. Therefore, care must be taken to inoculate plates with the same numbers of bacteria, keeping in mind that, no amount of the antimicrobial agent can prevent further growth if the bacteria reach a certain density (Petersdorf and Plorde, 1963; Petersdorf and Sherris, 1965 and Barry, 1980).

The combination of boric acid and pine oil showed a consistent effect on bacterial growth, being 1.5 to 7 times more effective in inhibiting bacterial growth compared to the control. In addition, with all species and beddings used the zone of clearing by the combination treatment was larger than for either boric acid or pine oil (Table 4).

Clearing zones in lawns of isolated ureolytic bacteria species derived from bedding materials contaminated with chicken manure (Fig. 17-19) showed different effects of the treatments. With the ureolytic bacteria isolates from contaminated woodshaving and sawdust, the growth inhibition was 2 X larger for pine oil and 3 X larger for boric acid and the combination in comparison to the control. These results are in agreement with the results reported in Fig. 15 and 16, where the combination was the most effective in ammonia production inhibition and was more effective than sawdust. The effect of these treatments on ammonia production might be due to their inhibition of ureolytic bacteria growth. The similarity in the results of the isolates of woodshaving and sawdust might be due to the close characteristic of these bedding materials; since the origin of both beddings is wood. With sand the zone of inhibition was 3, 2, and 4 times larger for pine oil, boric acid and their combination over the

Table 4. Effect Of Pine Oil, Boric Acid, And Their Combination On Ureolytic Bacterial Growth.[c]

No.	Isolate Source	Control	Pine oil	Boric acid	Combination
		Zone of Clearing in cm[b]			
1	Chick.wd[a]	0.17	0.35	0.52	0.52
2	Chick.sd[a]	0.17	0.35	0.52	0.52
3	Chick.sand	0.17	0.52	0.35	0.70
4	Pig wd	0.00	0.35	0.00	0.35
5	Pig sd.	0.00	0.70	1.40	1.57
6	Pig sand	0.52	0.87	0.70	0.87
7	Horse sand	0.00	0.17	0.35	0.70
8	Horse sd.	0.00	0.35	0.30	0.45
9	Horse wd.	0.10	0.35	0.52	0.70
10	Horse straw	0.00	0.70	0.70	0.87
11	Cow np.[a]	0.35	0.70	0.70	0.87
12	Cow sand	0.35	0.52	0.52	0.52

The final concentration of each treatment was, 16.8 % for boric acid, 6.7% for pine oil and 16.8% and 6.7% for the combination.

[a] Symbols for bedding: wd - woodshaving, sd - sawdust, np - newspaper.

[b] Distance is defined as the edge of the well to outer edge of no growth.

[c] All readings represent the mean of three determinations.

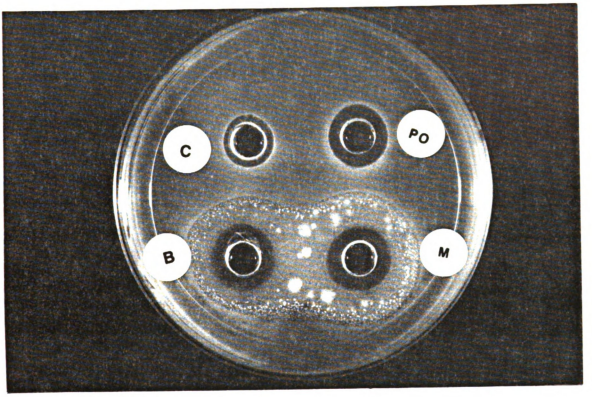


Figure 17: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from woodshaving bedding contaminated with chicken manure. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

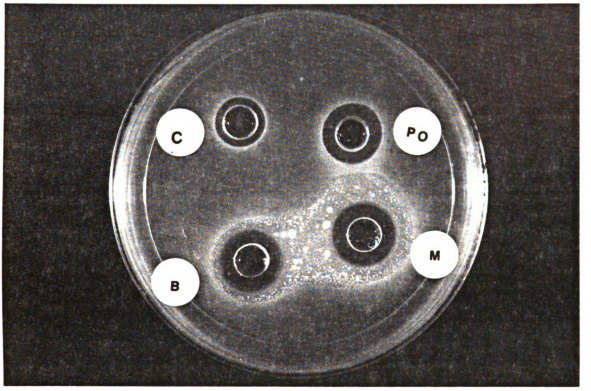


Figure 18: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sawdust bedding contaminated with chicken manure. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

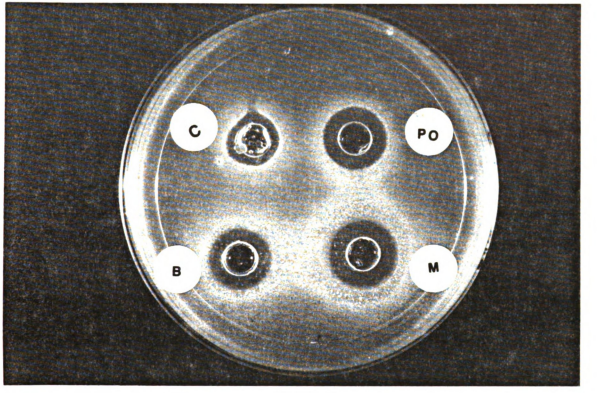


Figure 19: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sand bedding contaminated with chicken manure. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

control respectively.

Independent comparison among inhibition zones of ureolytic bacteria isolated from bedding materials contaminated with livestock urine and chicken manure are shown in Table 4. The difference in the inhibition zone sizes might be due to differences among species urine and beddings bacterial contents.

Growth inhibition of a ureolytic bacteria isolated from bedding materials contaminated with pig urine indicated that boric acid effect was totally suppressed in woodshaving (Fig.20). However, pine oil and the combination showed an equal effect in inhibiting bacterial growth (0.35). Therefore, it is concluded that the effectiveness of the combination was due to the presence of pine oil alone. Ureolytic bacteria isolated from sawdust contaminated with pig urine showed the best response to the treatments (Fig. 21). This result would suggest that boric acid and the combination (boric acid + pine oil) treatments were more effective with sawdust than with either sand (Fig. 22) or woodshaving (Fig.20). However, the ammonia production from woodshaving and sand was observed to be lower than sawdust (Fig. 12-14). This might be due to the different microbial contents (bacteria species and bacteria numbers) and different sensitivity of these microorganisms to the treatments.

With cultures derived from bedding materials contaminated with horse urine (Fig.23-26), boric acid and pine oil combination gave the best results; in which the inhibition zone was 7 X larger than the control. Boric acid was the most effective treatment against isolates from straw contaminated with horse

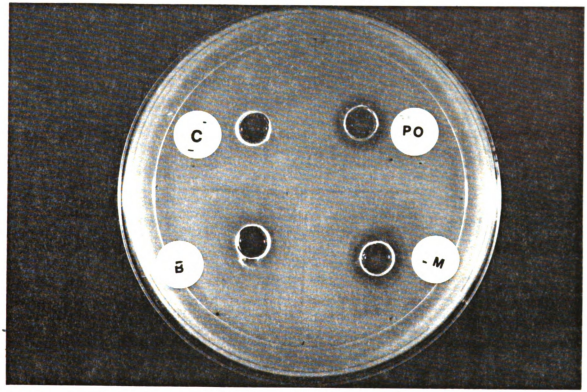


Figure 20: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from woodshaving bedding contaminated with pig urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

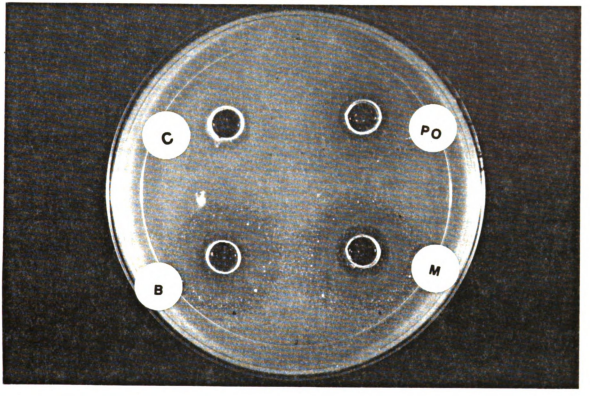


Figure 21: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sawdust bedding contaminated with pig urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

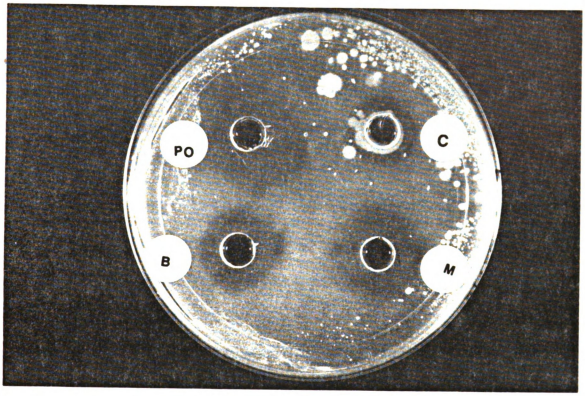


Figure 22: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sand bedding contaminated with pig urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

urine (Fig.24) and the least effective against isolates from sawdust contaminated with urine (Fig.23).

Bacterial cultures obtained from bedding materials contaminated with horse urine were inhibited best by the combination (M). These results are in agreement with the ammonia production study (Fig.9-11). Thus, the decrease in ammonia production of contaminated bedding treated with boric acid and pine oil might be due to the decrease in ureolytic bacterial growth.

Isolates recovered from bedding materials contaminated with cow urine (Fig.27-28) indicated that ureolytic bacteria isolated from newspaper contaminated with cow urine appeared to be more sensitive than the ureolytic bacteria isolated from sand contaminated with cow urine. With newspaper isolates, the inhibition zones were 2 to 2.5 fold larger in pine oil, boric acid and their combination compared to control. With sand isolates, the zones of clearing were 1.5 times higher for all treatments than control. This might be due to the high absorption of newspaper and to its low bacterial contents (Table 7). The combination provided the best results, however, pine oil and boric acid also provided a better inhibitory effect over the control. The combination effect could be the result of an interaction between pine oil and boric acid.

In conclusion, the result of these studies indicate that sand,as a bedding, and the combination (pine oil and boric acid), as a treatment, yielded the best results in terms of ureolytic bacteria growth inhibition. The effectiveness of treatments in sand might be due to its characteristic structure since inorganic

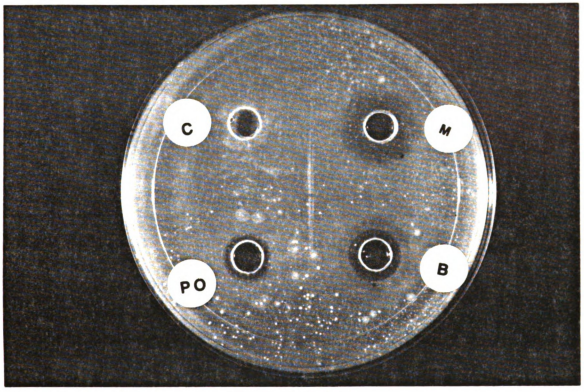


Figure 23: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sand bedding contaminated with horse urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

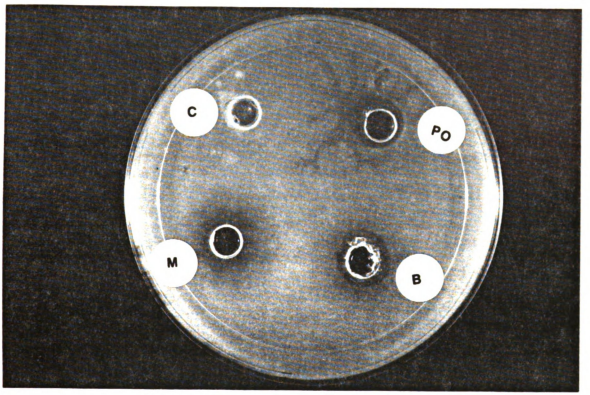


Figure 24: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sawdust bedding contaminated with horse urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

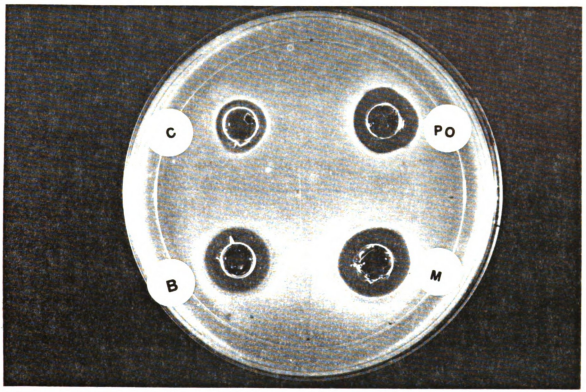


Figure 25: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from woodshaving bedding contaminated with horse urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

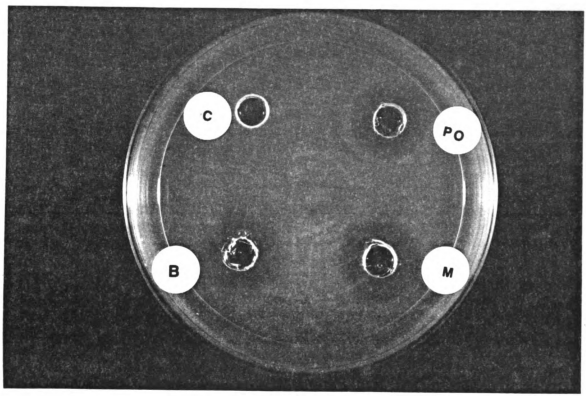


Figure 26: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from straw bedding contaminated with horse urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

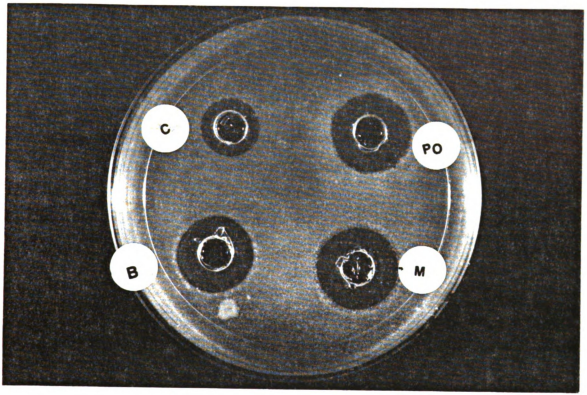


Figure 27: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolated from newspaper bedding contaminated with cow urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

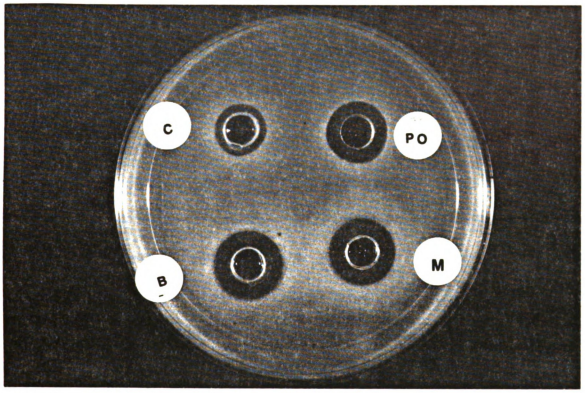


Figure 28: Effect of control (C), pine oil (PO), boric acid (B), and their combination (M) on growth of a ureolytic bacteria isolate from sand bedding contaminated with cow urine. The ureolytic bacteria was grown as a lawn onto nutrient broth agar plates.

material has little or non nutrient value to support the bacterial growth.

CHARACTERIZATION OF UREOLYTIC BACTERIA ISOLATED FROM BEDDING MATERIALS CONTAMINATED WITH LIVESTOCK URINE.

Although few bacteria are so morphologically remarkable as to make them identifiable without isolation, pure cultures are nearly always a necessity before one can attempt identification of an organism. It is important to realize that the single selection of a colony from a plate does not assure purity. This is especially true if selective media are used; live, but non-growing contaminants may often be present in or near a colony and can be subcultured along with the chosen organism. Therefore, non-selective media are preferred for final isolation, because they allow such contaminants to develop into visible isolation. Even with non-selective media, apparently well-isolated colonies should not be isolated too soon; some contaminants may be slow growing and may appear on the plate only after a longer incubation.

Generally, colonies from a pure culture that has been streaked on a solid medium are similar to one another, providing evidence of purity. Another criterion of purity is morphology since organisms from a pure culture generally exhibit a high degree of morphological similarity in stains or wet mounts.

Diverse bacterial species including various aerobes, facultative anaerobes and obligate anaerobes are responsible for urease production. Both Gram (-) and Gram (+) organisms may be urease producers (Collins et al., 1993), and they might be present in the bedding materials contaminated with livestock urine (Table 5). Therefore, it is important to isolate and characterize the

ureolytic bacteria in bedding materials contaminated with livestock urine.

Curtobacterium (Yamada and Komagata 1972) was isolated from sawdust, sand and straw contaminated with horse, cow, pig urine and chicken manure (Table 5). They are small irregular rods, cells become shorter to coccoid form in older culture. Branching and endospores are not found, and generally motile. Motile species show lateral flagellation. *Curtobacterium* are G(+), but old cells frequently lose the Gram (+) characteristic. They are obligately aerobic and show good growth on nutrient agar, and acid is produced slowly and weakly from glucose, fructose and some other carbohydrates. Colonies on nutrient agar are circular, smooth, entire raised, glistening, opaque and butyrous, and the color of the colonies depend on the species.

Morphologically, *Curtobacterium* strains show a bending-type cell division and often exhibit V forms. Rod forms are observed in young cultures, while coccoid forms predominate in older cultures, and branching is not observed. The size of the cells is 0.4-0.6 μm x 0.6-3.0 μm. Motility is observed in all species of *Curtobacterium* except *C. albidum* (Komagata and Lizuka; 1964; Lizuka and Komagata, 1965; Yamada and Komagata, 1972 a).

Most of the members of the genus *Curtobacterium* have been isolated from plants. *Curtobacterium citreum, Curtobacterium albidum*, and *Curtobacterium luteum* were isolated from rice (Komagata and lizuka, 1964). Therefore, the probable source of *Curtobacterium* is from the bedding. *C. flaccumfacies* is the only species regarded as a plant pathogen.

Table 5: Bacterial Species Isolated From Different Livestock Bedding Materials Contaminated With Livestock Urine Or Chicken Manure.

Bedding	Type of Bacteria	Tentative Genus/Species *
Chicken		
woodshaving	GN	*Salmonella*
sawdust	GP	*Curtobacterium luteum*
Pig		
sawdust	GP	*Curtobacterium luteum*
sand	GP	*Curtobacterium citreum*
woodshaving	GP	*Clavibacter michiganese*
Horse		
sawdust	GN	*Enterobacter gergoviae*
straw	GP	*Curtobacterium luteum*
woodshaving	GP	*Clavibacter michiganese*
sand	GP	*Curtobacterium luteum*
Cow		
sand	GP	*Curtobacterium luteum*
sawdust	GP	*Curtobacterium flaccumfacies*
corncobs	GN	*Klebsiella pneumoniae*
		Rahnella aquatilis

* Biolog (Release 3.50) was used for bacterial identification.

Enterobacter (Hormaeche and Edwards 1960) was isolated from sawdust horse urine (Table 5). They are straight rods, 0.6-1.0 μm wide 1.2-3.0 μm long, confirming to the general definition of the family *Enterobacteriaceae*. G (-), motile by pertirichous flagella. They are facultatively anaerobic and grow readily on ordinary media. Most clinical strains grow at 37°C; some environmental strains give erratic biochemical reactions at 37°C. They are widely distributed in nature; common in man and animals. *Enterobacter* is found only in sawdust contaminated with horse urine which suggests that urine is the source of this bacteria.

E. gergoviae of Gergoviae Highland; intended to pertain to the fact that the type strain isolated from samples taken during a urinary infection outbreak in Clermont-Ferrand University Hospital near Gergoviae Highland in France (Brenner et al., 1980). It is urease positive and occurs in various environmental sources such as cosmetics and water.

K. pneumoniae (Schroeter 1889; and Trevisa, 1887) was isolated from corncobs contaminated with cow urine (Table 5). It is normally found in the intestinal tract of man and animals, but in lower numbers compared with *E. coli*. It may be isolated in association with several pathological processes in man, e.g. infection of the urinary tract and respiratory tracts. In animals, *K. pneumoniae* may be isolated from metritis in mares and bovine mastitis.

Salmonella (Lignieres, 1900) were isolated woodshaving contaminated with chicken manure (Table 5). They are straight rods, 0.7-1.5 x 2.0-5.0 μm,

confirming to the general definition of the family *Enterobacteriaceae*. They are facultatively anaerobic, G(-) and usually motile. Colonies are generally 2-4 mm in diameter. They are pathogenic for humans, causing enteric fevers, gastroenteritis and septicemia; may also infect many animal species.

Classification of the isolated bacteria species indicated that *K. pneumoniae* and *E. gergoviae* are urease positive (Bergy's Manual Systematic Bacteriology). All other isolated bacterial species were urease negative (contains 0 to 10% urease activity). *K. pneumoniae* and *E. gergoviae* were isolated from sawdust and corncobs contaminated with horse and cow urine respectively (Table 5). Thus, urine could be the source of these bacteria.

MOST PROBABLE NUMBERS

Shape and morphology of the isolated ureolytic bacteria (Table 6) were found to be in agreement with the reported data. Urine contains some endogenous nitrogen resulting from small inefficiencies of the cycling of amino acids from one protein to another in the animal. This endogenous nitrogen loss is proportional to the metabolic size of the animal (Asplund, 1971). When urea is fed to ruminants some urea and some ammonia is absorbed by the rumen, filtered by the kidney, and lost in the urine (Asplund, 1971).

About 70% of the nitrogen comes from the urine fraction of the mixture in poultry manure (White et al., 1944). A relatively large proportion of the total nitrogen of fresh poultry manure is in a form of uric acid, an insoluble material which appears visibly as the "white cap" on poultry droppings. Leibholz

Table 6: Shape And Morphology Of Isolated Ureolytic Bacteria Examined Under Phase Contrast Microscopy.

Species	Bedding	Shape and morphology
Chicken	sawdust	rods, motile, short and long chains.
	woodshaving	rods, motile, short and long chains.
Horse	sawdust	rod, short chain.
	straw	rods, single and pair groups.
	woodshaving	rods, single and pair groups.
	sand	rods, short groups.
Cow	sand	rods, single and pairs groups.
	sawdust	rods, motile and short chains.
	corncobs	rods, single and short chains.
Pig	woodshaving	rods, short chains.
	sand	rods, motile, single and double chains.
	sawdust	rods, short chains.

(1961), reported that there is about 39% of the nitrogen as uric acid in one sample of poultry manure. Uric acid makes up about 80% of the nitrogen in poultry urine (O'Dell et al., 1960). On the other hand, from studies on chicken with modified ureters, revealed that about 55 to 58% of fresh poultry manure was urine (Dixon, 1958).

Chicken manure contained the highest ureolytic bacterial numbers ($4.6x10^5$) (Table 7). Nodar et al (1990) reported that the numbers of total viable microorganisms in poultry excreta was about 10^8 per g dry excreta. Aerobic bacteria represented a small proportion of total bacteria with counts of 10^6 CFU/g excreta. Among the microorganisms related to the nitrogen cycle, the proteolytics and the ammonificants were the most abundant, with densities of 10^8/g excreta. The densities of anaerobic-free living nitrogen-fixers and denitrifiers, both with values of 10^5/g dry excreta, were also relatively high. Whereas the numbers of ammonium oxidizers are about 10^4 per g dry excreta. Nitrite oxidizers and aerobic free-living nitrogen-fixers were relatively low . The high ureolytic bacterial number in chicken manure ($4.6.x10^5$) is in accordance with the fact that almost all the inorganic nitrogen in poultry excreta is ammonium. Therefore, the largest bacteria groups, by far, were those that carry out the breakdown of proteins and the ammonifying organisms.

In all species ureolytic bacterial number was the highest in contaminated bedding followed by urine (Table 7). Surprisingly, ureolytic bacterial number in sand bedding has the highest number ($1.1x10^3$) in horse, cow and pig (Table

Table 7: Ureolytic Bacterial Numbers In Urine And Different Bedding Materials Contaminated With Livestock Urine Or Chicken Manure.

Species	Ureolytic Bacteria #	Bedding/urine/manure
	Bacteria numbers/100 ml media	
Pig	2.4×10^2	sand + urine
	2.4×10^2	sawdust + urine
	2.4×10^2	urine only
Cow	2.4×10^2	sawdust + urine
	2.4×10^2	urine only
	2.3×10	straw + urine
	2.3×10	newspaper + urine
	2.3×10	corncobs + urine
	2.3×10	woodshaving + urine
	1.1×10^3	sand + urine
Horse	1.1×10^3	sand + urine
	2.8×10	sawdust + urine
	1.2×10^2	urine only
	2.1×10^2	woodshaving + urine
	2.4×10^2	straw + urine
Chicken	4.6×10^5	sawdust + manure
	4.6×10^5	woodshaving
	4.6×10^5	manure only

7). However ammonia production from sand contaminated with livestock urine was very low. Hogan et al. (1989) reported greater bacterial numbers in sand over sawdust and straw. Organic bedding materials may provide an exogenous source of essential nutrients for maintenance of bacterial populations which is not the case in an inorganic bedding (sand). These results are in agreement with the observations of Bramley and Neave (1975) that sand is a suitable alternative for straw, woodshaving and sawdust.

THE INHIBITORY EFFECT OF BORIC ACID, PINE OIL AND THEIR COMBINATION ON THE GROWTH OF PURE CULTURES OF MASTITIS CAUSING BACTERIA

There is evidence that the growth of microorganisms in bedding material is associated with a higher incidence rate of clinical mastitis (Bramley and Neave, 1975 and Hogans et al., 1989). However, in herds with low colony counts in bedding material, substantial variation exists in the incidence rate of clinical mastitis.

Management practices that reduce the exposure of mastitis causing pathogens to the teat end should result in reduced rates of new intramammary infections. During lactation exposure of teat ends to coliforms and environmental *streptococci* occurs primarily between milking. Bedding materials have been implicated as primary source of environmental pathogens during the intermilking periods (Eberhart and Buckalew, 1972; Eberhart et al., 1983, and Rendos et al., 1975). Field surveys have shown extreme variation among rates of clinical mastitis and bacterial counts in bedding (Bramley, 1982, and Carroll

and Jasper, 1978). The sequence of events leading to infection is usually one of progression from pathogen contamination of teat skin to invasion of the teat canal and proliferation within the mammary gland. Theoretically, any factor that increases the number of pathogens on apical skin will increase the incidence, while factors that decrease pathogen presence will reduce the incidence of infection.

Diffusion susceptibility testing (disc test), depends on the ability of paper disc impregnated with an antimicrobial agent to release it into an agar medium, thus creating a circular diffusion gradient of the agent around the disc. The culture plate was covered with an inoculum of the test organism prior to the placement of the disc, and zones of inhibition of bacterial growth then appear around the disc after overnight incubation. The type of paper used, thickness and diameter of the disc, and volume of the treatment used to impregnate the disc do not effect the zone size. Similarly, the depth of the medium, age of the agar and moisture of the medium have little effect on zone size. The size of the inoculum has actually been found to be the single most important variable influencing the size of the inhibition zone because the position of its border is determined when the critical cell mass is attained (Barry, 1980). Obviously, the more microorganisms in the inoculum, the greater the opportunity for generating visible growth before the antimicrobial agents reach an inhibitory concentration at a given distance from the disc. Conversely, the fewer organisms used to seed the plate, the lesser their chance for producing visible growth at that

distance before inhibition takes over. No amount of the antimicrobial agent can prevent further growth if the bacteria reach a certain density (Petersdorf, and Plorde, 1963; Petersdorf and Sherris, 1965 and Barry, 1980), so use of a heavy inocula will produce smaller inhibition zones.

Exclusion of NaOH from the treatments combination reduces the inhibition zone size as shown in Fig. 29-34, this data (in Fig. 29-34) is also summarized in (Table 8). Bramley and Neave (1975) suggested that maintenance of low levels of coliform contamination of bedding is the only method of control which can be effective. In addition Jasper (1980) reported that contaminated bedding materials increased coliforms populations during the in vitro trials. Therefore, frequent removal of contaminated bedding was recommended to decrease bacterial numbers in bedding. The pH of all treatments without NaOH ranged from 3.5 to 5.1. On the other hand the pH of the treatments which included NaOH ranged from 8.7 to 12.5. This difference in pH explains the better effect of treatments that included NaOH over those that did not contain it.

Mastitis is determined by environmental factors, cow related factors and pathogens. Therefore, several attempts have been made to study the effects of different hygine treatments on mastitis causing bacteria and as a result on mastitis incidence. Murdough and Pankey (1993) tested the effect of fifty-seven teat dip formulation on germicidal activity. Among the microorganisms studied were *E.coli* and *S.aureus*. Their results showed that 30% and 60% of

these products had mild effect on *S.aureus* and *E.coli* respectively. A common teat dips used in dairy barns contain iodophor or chlorohixidine digluconate, these dips showed significant potential for skin irritation. Therefore other germicidal formulations are under investigation (Sears et al 1992). All these studies aim to protect the mammary gland against the mastitis causing bacteria and most of these studies focus on *E.coli* and *S.aureus*. This study showed that pine oil, boric acid and their combination are effective in limiting the mastitis microbial growth. Even when NaOH was omitted bacterial growth was affected. Without NaOH, boric acid was the most effective treatment. When NaOH was added the combination was the most effective. This is due to the neutralization effect of NaOH (strong alkaline) on Boric acid (weak acid). Pine oil contains high levels of terpene volatiles, including some oxygenated monoterpenes such as terpineol and limnonene (Nijholt, 1980) , which has antimicrobial properties. Therefore, it wasn't surprising to observe microbial growth inhibition when pin oil was used.

The combination (PO + B), with NaOH showed more consistent results in terms of inhibiting the bacterial growth. However, boric acid showed better results with *Staphylococcus aureus*. These results indicate that the treatments are effective in inhibiting the mastitis causing bacteria growth. The rates of environmental mastitis increases with increasing teat end exposure topathogens in bedding. Hogan et al (1989) reported a significant relationship among total rated of gram negative-bacteria and *Klebsiella* species in lactating cow bedding.

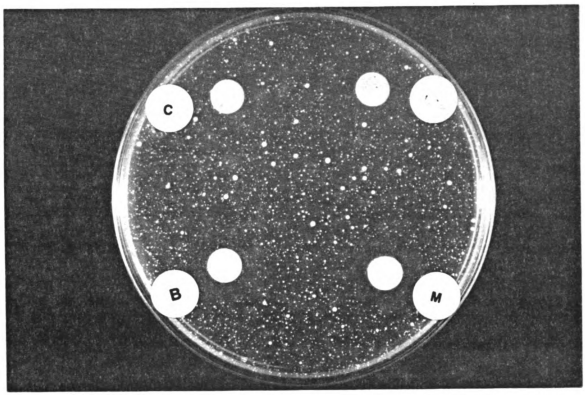


Figure 29: Effect of control (C), pine oil (PO), boric acid (B) and their combination (M) on growth of *Staphylococcus aureus*. Treatments were prepared as previously described on page 30. NaOH was omitted from all treatments and substituted by its exact amount of sterile water.

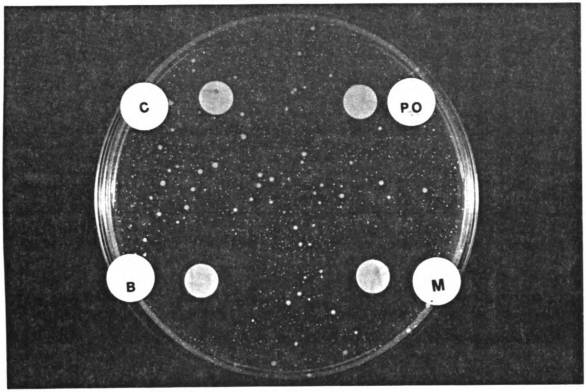


Figure 30: Effect of control (C), pine oil (PO), boric acid (B) and their combination (M) on growth of *Staphylococcus aureus*. Treatments were prepared as previously described on page 30. All treatments included NaOH.

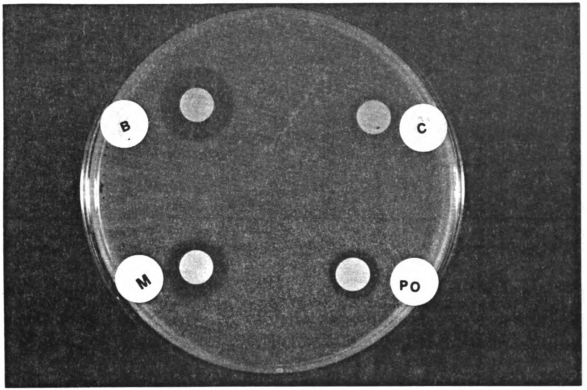


Figure 31: Effect of control (C), pine oil (PO), boric acid (B) and their combination (M) on growth of *Klebsiella pneumonia*. Treatments were prepared as previously described on page 30. NaOH was omitted from all treatments and substituted by its exact amount of sterile water.

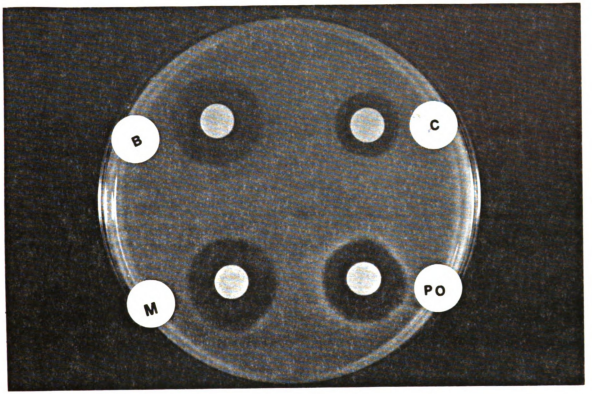


Figure 32: Effect of control (C), pine oil (PO), boric acid (B) and their combination (M) on growth of *Klebsiella pneumonia*. Treatments were prepared as previously described on page 30. All treatments included NaOH.

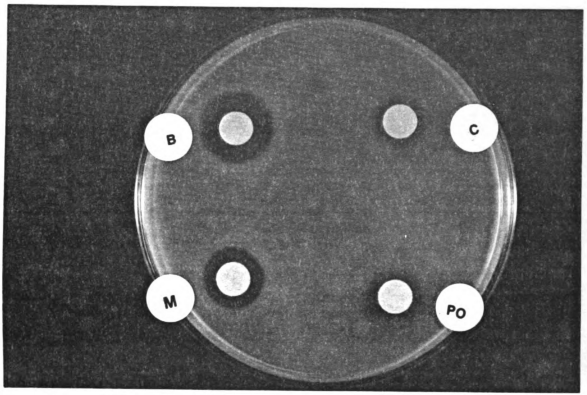


Figure 33: Effect of control (C), pine oil (PO), boric acid (B) and their combination (M) on growth of *E.coli.* Treatments were prepared as previously described on page 30. NaOH was omitted from all treatments and substituted by its exact amount of sterile water.

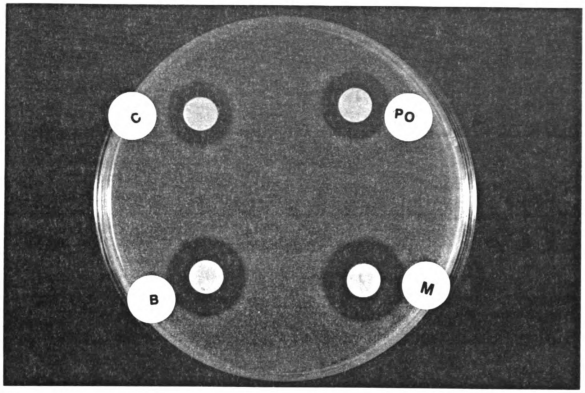


Figure 34: Effect of control (C), pine oil (PO), boric acid (B) and their combination (M) on growth of *E.coli*. Treatments were prepared as previously described on page 30. All Treatments included NaOH.

Table 8: Inhibition Of Growth Of Mastitis Bacteria.

Species	Control	Pine Oil	Boric Acid	Combination
	Zone of Clearing (cm)[a]			
Staphylococcus aureus	0.00	0.00	0.90	0.70
Staphylococcus aureus[b]	0.00	0.20	0.30	0.50
Klebsiella pneumoniae	0.30	0.50	0.70	0.70
Klebsiella pneumoniae[b]	0.00	0.20	0.50	0.30
E. coli	0.40	0.45	0.60	0.70
E. coli[b]	0.10	0.10	0.50	0.30

[a] Each value is a mean of three determinations and all values are in cm.

[b] Indicates that NaOH was omitted from the treatment and substituted with an equivalent amount of sterile water.

CONCLUSION

1- Daily measurements of ammonia production from bedding materials contaminated with different livestock urine indicated a significant effect of the treatments boric acid, pine oil and their combination in reducing ammonia production. Kitagawa Tubes have proven to be efficient in measuring ammonia.

2. Pine oil (P), boric acid (B) and their combination (M) showed different levels of inhibition depending on urine source and bedding material used. The combination was the most effective treatment. This could be due to the combined effect of (P) which has an antimicrobial property and (B) which is a known urease inhibitor.

3. Different bedding materials (organic and inorganic) showed different effects on ammonia production. The two properties of bedding likely to exert the greatest influence on odor production are its absorption and physical structure. Woodahaving showed very low ammonia production, this seemed to be due to the fact that it either contains an unknown inhibitory substance (s) or other properties that caused a decrease in ammonia production. In general, sawdust and sand showed the maximum response to the treatment.

4. Because of the variation in urine and manure characteristics among species, urine source affected ammonia production. Ammonia production was higher from urine high in nitrogen (N) content (i.e. cow and horse) and lower from urine low in (N) content (i.e. pig and chicken).

5. Treatments showed a significant inhibitory effect on ureolytic bacterial growth which might explain the mechanism of action of the treatments. In addition, the treatments evaluated showed a similar effect on mastitis causing bacteria, such as *Staphylococcus aureus* and *Klebsiella pneumoniae*.

6. Since the data obtained in this research indicates that ammonmia production, from livestock contaminated bedding, started to increase at the second day and peaked at the fifth day, applying the treatments once the bedding is spread on the floor would be highly profitable. Thus inhibiting the bacterial activity and reducing ammonia production.

In general, the evidence indicates that the odor from a livestock building increases as waste accumulates in the building. The only waste management technique which can, without question, reduce livestock building odor production is removal of waste from the building. It is not clear whether the increase in odor as waste accumulates is caused by the extra volume of waste, by the biological changes taking place as it ages and anaerobic conditions develop, or both. If frequent waste removal is not practical, there is evidence that inhibiting the development of anaerobic conditions and inhibiting urease activity may reduce ammonia production.

Several areas of research on livestock odor production need further investigation:

1. The mechanism of action of odor inhibitors (i.e, studies on the effect of odor inhibitors on urease enzymatic activity and gene expression).

2- On the prospect of feed and manure additives, there is a lack of standardized test and evaluation procedures for the commercial products.

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